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STUDIES ON AMPHIBIAN METAMORPHOSIS. III. THE INFLUENCE OF THE ANNULAR TYMPANIC CARTILAGE ON THE FORMATION OF THE TYMPANIC MEMBRANE

(Five plates and four figures)

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INTRODUCTION

THE formation of the tympanic membrane and associated structures during the metamorphosis of the frog larva has been known for some time. In general, the following embryological developments and transformations occur in the formation of the middle ear of the adult frog. A posterior and dorsal process of the metapterygoid region of the quadrate is formed, and its distal end finally constricts off to form a cartilaginous ring, the annular tympanic cartilage (*ATC*, Fig. A). The annular tympanic cartilage gradually shifts posteriorly and in time becomes associated with the tympanic cavity beneath connecting with the Eustachian tube, and with the lamina propria which covers its lateral surface. Near the end of metamorphosis, and at a time when the tail is completely resorbed (Fig. B), the adult structures of the middle ear are quite well formed. The annular tympanic cartilage appears slightly circular or slightly oval in shape, and is completely covered by the translucent lamina propria. The columella extending through the tympanic cavity is seen to touch the lamina propria near its center. The external appearance of the tympanic membrane at this time is represented in Figure C.

Histologically, the tympanic membrane consists of three distinct layers. The lamina propria must be considered as the foundation layer and corresponds in shape and area to the annular tympanic cartilage to which it is attached. Internally, an epithelial layer, continuous with the mucous membrane of the tympanic cavity, is closely applied to the lamina propria; while the third and

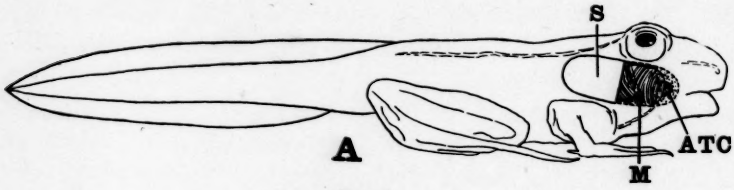
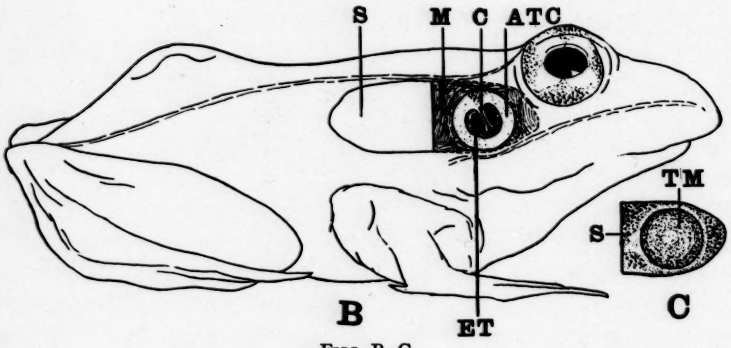


FIG. A



FIGS. B, C

external layer consists of modified integument. This latter layer is visible externally as a conspicuous, almost circular area, brownish in color and slightly indented. Its formation is gradual from normal integument and is the result of definite histological transformations.

The present paper deals, primarily, with the factors responsible for the development of this integumentary layer of the tympanic membrane[1] during metamorphosis. The fundamental question involved here is whether the thyroid hormone initiates the integu-

[1] The term "tympanic membrane," as used throughout the present paper, refers only to the *integumentary* portion of the membrane.

mentary changes responsible for tympanic membrane formation directly, through the blood stream, or whether intermediate influences are set in operation which produce the same end result. In either case the specificity of the skin for tympanic-membrane formation constitutes a second problem. That is to say, is the skin which normally develops into tympanic membrane hereditarily specific in this respect or does all integument inherit such potentialities, the skin of the tympanic membrane region undergoing the histological transformation due solely to its association with other structures of the middle ear.

As far as the writer is aware, the present paper constitutes the first attempt to explain these points regarding the development of the tympanic membrane of the anuran. Previous workers have concerned themselves, chiefly, with the histology of the adult membrane. Of these, Moldenhauer (1878) has pictured and described, quite fully, the conditions found in *Rana esculenta* and *Rana temporaria*. More recently, Tokura (1925) describes the peculiar fibrous construction of the dermal layers in the tympanic membrane of *Rana nigromaculata*, a variety of *Rana esculenta*. The correspondence of *Rana pipiens* in this respect will be taken up in a later section of this paper.

The methods of attack followed in the present work were briefly as follows: Skin transplants were made from the side and back of the tadpole to the tympanic membrane region, after first having removed the area of integument which normally forms tympanic membrane. Reciprocal grafts of skin from the tympanic membrane area transplanted to the back and side were also made on normal tadpoles. During metamorphosis, the possible formation of tympanic membranes in foreign integument or in skin of the tympanic membrane region transplanted to an abnormal location could be observed, and light thrown on the question of a possible self-differentiating system being present in the skin of the tympanic membrane region. Other series of operations involved the extirpation of annular tympanic cartilages at early and late developmental stages and their transplantation under the normal skin of the side and back. The possible influence and relationship of this structure to tympanic membrane formation was thus determined. These

operations, which will be described in detail in later sections of the present paper, included the following series of experiments:

1. Autoplastic skin transplantations (42 cases): Skin grafts from tympanic membrane region of the right side interchanged with skin grafts from the back.

2. Autoplastic skin transplantations (34 cases): Skin grafts from tympanic membrane region of the right side interchanged with skin grafts from the side.

3. Autoplastic annular tympanic cartilage transplantations (52 cases): Annular tympanic cartilage transplants beneath side skin of tadpole.

4. Autoplastic annular tympanic cartilage transplantations (65 cases): Annular tympanic cartilage transplants beneath back skin of tadpoles.

MATERIALS AND METHODS

Full-grown larvae of *Rana pipiens* were employed for the operations. They were collected during the month of July from a small pond in the vicinity of Iowa City, Iowa. Because of the ease of collecting thousands at a time, selection of the healthiest-appearing specimens was possible and helped considerably in lowering the mortality rate of those employed for operative work.

The selected specimens were placed in suitable aquaria in the laboratory and fed regularly on a diet consisting largely of *Spirogyra*. *Spirogyra*, as a preoperative food, has been found very satisfactory by the writer for all species of frog larvae. The animals fatten up within a few days, and apparently increase their vigor, as compared with tadpoles fed on other diets. This is especially useful where the tadpoles are to be metamorphosed, artificially.

In selecting tadpoles for operative work, only those of a certain size were used. For certain of the transplant operations, tadpoles in various stages of metamorphosis were selected; while in other cases, the host animals were fully grown, normal tadpoles on the verge of metamorphosis. Such individuals invariably underwent the first stages of metamorphosis within 2–5 days following the operation.

The operations were mainly carried out under anesthesia. For this a 0.05 per cent aqueous solution of chloretone (trichlortertiary-

butyl alcohol [Parke, Davis & Co.]) was employed. In general, slow anesthesia was found to be more satisfactory than rapid, although the individual resistance to the anesthetic was subject to some variation. It was found, however, that if the animals were anesthetized for about 30 seconds longer than necessary to inhibit movement, the best results would be obtained. The purpose of thus regulating the anesthetization period was to induce a period of anesthesia just sufficient to carry out the operation and allow sufficient time for the grafts to adhere.

Postoperative treatment consisted in placing the animals in shallow water so that the grafted area and wound incisions were exposed to the air for at least 15 minutes. Such exposure resulted in the congealing of the lymph and blood and the consequent adhering of the grafts sufficiently to allow total immersion of the animal. Proper anesthetization resulted in the recovery of animals soon after such immersion. The operated animals were isolated in small battery jars and fed daily with *Spirogyra*. The changing of the aquarium water each day assisted materially in reducing the mortality. Case histories for all operated animals were maintained until the individuals died or were killed for histological examination.

FORMATION OF THE NORMAL TYMPANIC MEMBRANE

1. MACROSCOPIC SEQUENCE OF EVENTS

The first external indication of tympanic membrane formation consists of the appearance of a small oval area just beneath and slightly posterior to the eye. The area appears slightly reddish in color as compared with the surrounding integument, and is of a uniform shade. Its boundaries are not marked off very distinctly, however, the gradation into normal integument being a gradual one at every point. This first indication of the future location of the tympanic membrane occurs at a rather late stage of metamorphosis. In an average 80 mm. tadpole, both fore legs have emerged, the hind legs are approximately 40 mm. in length, while the tail is about one-half atrophied. The tadpole has assumed the typical spotting of the adult frog and is seen to spend considerable time floating on the surface of the water.

As development proceeds, the small oval area tends to assume

a more circular shape, although still retaining its reddish tinge. The anterior and posterior margins, however, are now seen to be somewhat indistinctly bounded by dark grayish lines, the dorsal and ventral boundaries still exhibiting a gradual gradation into the normal integument. Further development results in the approximation of these lines dorsally, so that the area as a whole soon assumes a triangular shape and shifts further posterior. Acccompanying this configuration, the skin is seen to assume a deeper shade of red, and the area as a whole is quite distinctly marked off. The texture of the skin is still typically tadpole-like, however, and shows no signs of indentation. At this stage the tail is reduced to a mere 10 mm. stump, and the rapidly metamorphosing tadpole has assumed the sitting posture of the adult frog.

The succeeding steps now follow quite rapidly. The area soon becomes more oval in shape and, with the exception of a small, dorsal, anterior region at the periphery adjacent to the eye, is distinctly marked off from the surrounding integument. At this stage, however, two characteristics of the fully formed membrane make their appearance, namely, indentation and texture. The indentation is very slight at first and is noticeable only when the animal is viewed obliquely and under a strong light, since the depression is slight and quite gradual at the periphery. The texture of the skin is now quite different from that of normal integument. The reddish, pigmented appearance has been replaced by a finely mottled or stippled effect; and the whole membrane is distinctly light brown in color, except for a narrow gray zone at the periphery. This condition obtains when most of the external transformations of the animal are practically completed, the tail, for example, being but a small black stump about three mm. in length.

Following this, the area tends to become more circular in shape and distinctly outlined at all points. The color deepens somewhat; and by the time the tail is completely resorbed, it resembles in shade the membrane of the full-grown frog. The area is now also quite strongly indented and has shifted posteriorly and dorsally to approximately its final orientation. The external changes from this period on are slight and consist chiefly in a uniform enlargement in diameter and a gradual change in color from brown to grayish

brown. The size of the tympanic membrane increases gradually and in proportion to the growth of the adult frog.

2. HISTOLOGICAL SEQUENCE OF EVENTS

Sections made through the skin of the tympanic-membrane region at succeeding stages of metamorphosis show quite clearly that the various integumentary layers undergo an orderly process of histolysis and transformation which results in the formation of the typical tympanic membrane of the adult frog. The process is a gradual one and, once initiated, appears to progress at a uniform rate. For convenience of description, however, it may be divided into three fairly distinct transformations as pictured in Figures 1, 2, 3, and 4, Plate I.

The normal integument, as shown in Figure 1, is maintained up to a time slightly preceding the appearance of the first macroscopic sign of tympanic membrane formation. The integument is normal in all respects as compared with other side skin—skin glands pigment cells, and the various layers being fully represented.

The first signs of tympanic membrane formation are concerned with the structure of the stratum compactum layer. The layer loses its convoluted appearance, and the individual fibrils composing it tend to straighten out, so that they are more or less parallel to the surface. Considerable disarrangement of the fibril bundles next takes place, so that the layer as a whole presents a very disorganized picture (*SC*, Fig. 2). The long spindle-shaped nuclei of the normal stratum compactum appear less frequently and are either lost at this time or, more probably, covered over by the dissociating fibrils. Paralleling this dissociation, lymphocytes make their appearance and apparently migrate from the subcutaneous connective tissue into the compactum layer. They may be found at all levels but are particularly abundant where the two layers meet and are apparently engaged in removing dissociated fibrils at this point. At this stage of transformation all other layers of the skin are intact, so that the integument appears normal when viewed externally.

The dissociation of the stratum compactum progresses rapidly (Fig. 3) and in time all signs of unification are lost. The fibrils appear to group together into fused bundles and invade the subcutaneous

PLATE I

FIGS. 1–4.—Sections through the tympanic membrane region of the integument of a tadpole, showing the successive cytological steps leading up to the formation of the tympanic membrane (*Rana pipiens*). *E*, epidermis; *SS*, stratum spongiosum; *SC*, stratum compactum; *SB*, subcutaneous connective tissue; *P*, pigment masses; *L*, lymphocytes; *V*, blood vessels; *G*, glands; *VF*, vertical fibers. ×110.

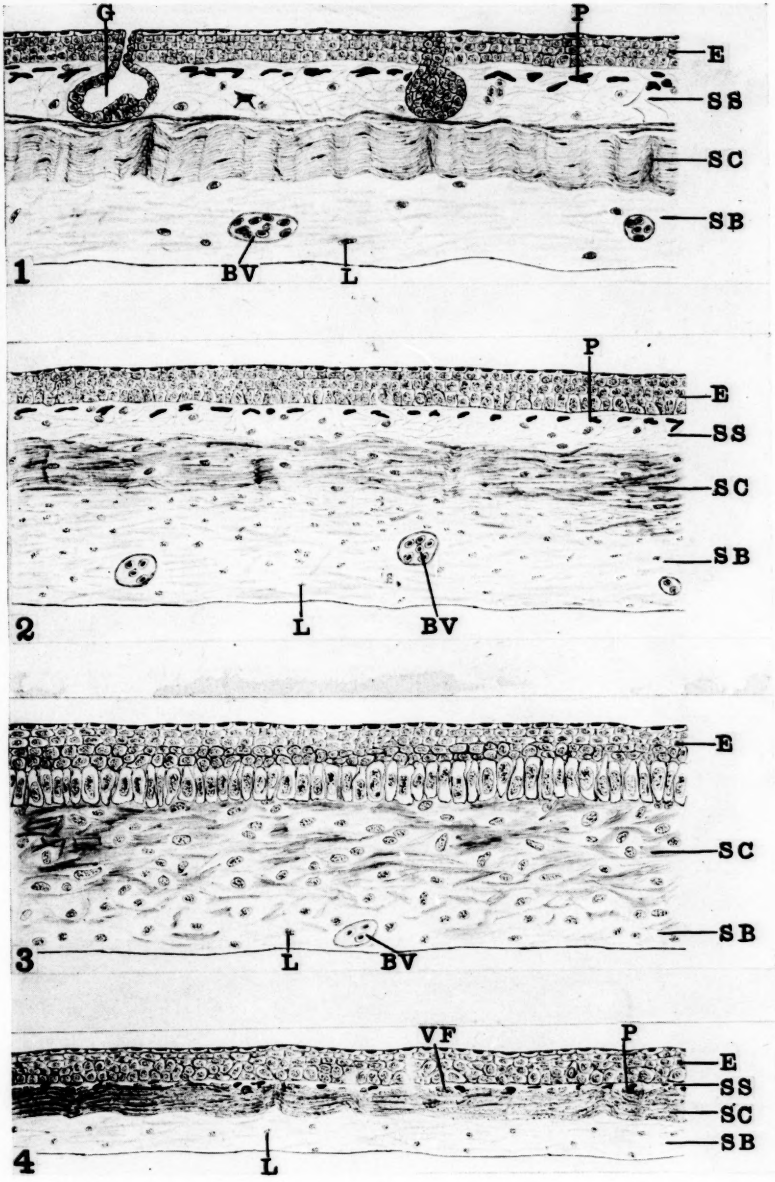
FIG. 1.—Normal integument of tympanic membrane region.

FIG. 2.—First cytological changes. Fibrils of stratum compactum lose their convoluted, wavy appearance and begin to dissociate. Lymphocytes begin to invade the area quite heavily.

FIG. 3.—A considerably more advanced stage. The epidermis has increased in thickness, and the cells of the basal layer have hypertrophied. The stratum compactum is broken up into bundles of dissociating fibrils, which obscure the stratum spongiosum. Lymphocytes are large and active.

FIG. 4.—Appearance of the tympanic membrane at the end of metamorphosis. A narrow stratum spongiosum has appeared, while the epidermal and subcutaneous layers are reduced in thickness. The stratum compactum is composed of granulated, horizontal, and vertical fibers. Lymphocytes become scarce.

PLATE I



connective tissue. At this stage the lymphocytes are very numerous and appear quite large in size. The stratum spongiosum is entirely obliterated, and the pigment cells and glands characteristic of its normal structure are absent. In brief, all space below the epidermis, with the exception of the lower levels of the subcutaneous connective tissue, is filled with the dissociating bundles of stratum compactum fibrils. The epidermis at this time exhibits cytological changes for the first time. The cells of its basal layer hypertrophy greatly and compose nearly one half of the thickness of this layer. The cells are columnar in shape and contain large nuclei and vacuolated spaces. This condition, together with the absence of subepidermal pigment, no doubt accounts for the reddish tinge of the skin when viewed externally. The remaining cells of the epidermis appear to increase in number, so that the epidermis is one or two layers thicker at this time. The size of these cells remains quite constant, however.

Subsequent changes in the skin are concerned with the building up of structures typical of the adult tympanic membrane. The basal cells of the epidermis are either replaced by smaller cells or reduced in size (Fig. 4), so that the resulting epidermis appears normal again. The stratum compactum assumes a wavy appearance again, although it is seldom thrown into the pronounced convolutions typical of normal skin. The fibrils, however, appear to be larger in diameter and are grouped into loose bundles. Typical stratum compactum nuclei are absent, while scattered lymphocytes may still be found. Numerous small granules appear to be distributed quite uniformly throughout the layer; and it is just possible, though unlikely, that they represent the cut ends of fibrils. The resultant appearance of the stratum compactum is one not to be confused with the corresponding layer of the normal skin. Aside from the fact that it is little more than one-half the thickness of the latter, it always has a deeper staining reaction and is more closely knit together. The exact nature of its structure is interesting to consider at this time. According to Tokura (1925), it is composed of two layers of fibrous elements, viz.: an inner thick region of horizontal fibers and an outer region of smaller fibers running vertically, which cross with those of the inner region to form a complex network. This, in general, the

writer found to be true in *Rana pipiens*. Several of the outer vertical fibers are represented in cross-section in Figure 4, *VF*. At this time a thin stratum spongiosum layer makes its appearance, although it is apparently absent at certain points where the stratum compactum is in close contact with the epidermis. Where it is present, pigment cells soon develop; and occasional glands, always small in size, occur distributed unequally at different levels of the membrane. The subcutaneous connective tissue is by this time quite normal, although considerably decreased in thickness, as compared with that of normal skin. The resulting histological picture (Fig. 4), therefore, is that found at the close of metamorphosis and at which time the tympanic membrane appears as a well-defined, light-brown area, externally. In total thickness the integument is now less than one-half that of the normal integument from which it was derived.

3. UNION OF THE VARIOUS LAYERS OF THE TYMPANIC MEMBRANE WITH THOSE OF THE SURROUNDING INTEGUMENT

In the newly metamorphosed tadpole, the junction point of the tympanic membrane with the surrounding integument, although distinguishable histologically, is not very pronounced at first. As the frog develops, however, a comparatively narrow zone is evident between the two tissues which defines the periphery of the tympanic membrane histologically, and corresponds roughly in position to the rim of the annular tympanic cartilage beneath (*X*, Fig. 9, Plate III). In the fully grown frog (Fig. 5, Plate II) the following points are distinguishable in this region. The epidermis is seen to bend sharply downward, chiefly at the expense of the stratum spongiosum. This is evident, externally, as marking the boundary of the indented membrane. The epidermis from this point is also gradually reduced in thickness to conform with that of the tympanic membrane.

The stratum spongiosum of the normal integument narrows quite strikingly to join with the much narrower stratum spongiosum of the tympanic membrane. It is interesting to note here, that the skin glands, which are quite small and few in number in this layer of the tympanic membrane, may be found normal in size and abundance in the adjacent integument within a short distance of the union area. The relationship of the two compactum layers is not so clear, however. In general it may be said that most of the fibers

(horizontal fibers of Tokura [1925]) gradually merge with the fibrils of the normal stratum compactum. The latter appear to run past the rim of the annular tympanic cartilage, however, and merge with the tympanic membrane fibers a short distance in from the point of indentation of the epidermis.

That most of the tympanic membrane fibers connect with the stratum-compactum layer of the normal skin seems quite certain. If the tympanic membrane has been sectioned horizontally, however, a small portion of the outer stratum compactum of the tympanic membrane is seen to spread out, and the individual fibers appear to run into the stratum spongiosum of the normal integument (*X*, Fig. 5, Plate II). The junction area of these fibers is small in extent and occurs just beneath the indentation of the epidermal layer. These fibers generally take a deeper stain and are probably of the vertical layer as described by Tokura (1925). The subcutaneous connective tissue of the two integuments is continuous and undergoes no transformation except a slight decrease in thickness. When the cross-section has been made to include the annular tympanic cartilage (Fig. 9, Plate III), the subcutaneous connective tissue is nearly obliterated where the rim of the cartilage comes in close approximation to the stratum compactum. This is not unusual, since the subcutaneous connective tissue is subject to considerable variation in depth in different regions of the tympanic membrane.

SPECIFICITY OF THE INTEGUMENT

1. PURPOSE OF THE SKIN TRANSPLANTATIONS

The development of the tympanic membrane at a certain stage of metamorphosis, and in a location involving always approximately the same area of integument, suggested one of two alternatives regarding the specificity of integument in this regard. Either the area of integument involved is hereditarily specific in its ability to transform into tympanic membrane under metamorphic influences or else non-specific integument, by virtue of its association with structures of developing middle ear, is capable of so transforming. It is clear that if the former condition existed, then transplantation of such integument to other regions of the tadpole should not inhibit its development into tympanic membrane during metamor-

PLATE II

FIG. 5.—Section through the periphery of a newly formed tympanic membrane, showing its union with the surrounding integument. The tympanic membrane is pictured on the right side, while the normal integument is shown at the left. *X*, fibers of stratum compactum merging with stratum spongiosum of normal integument. ×110.

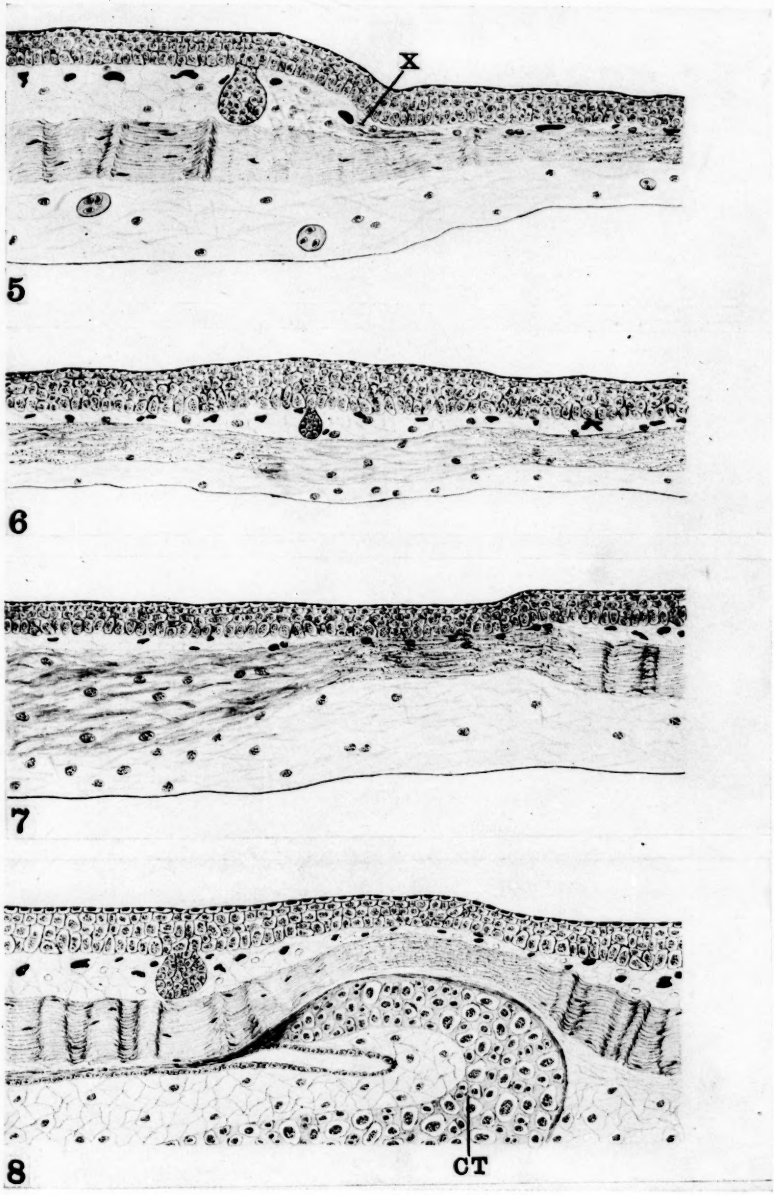
FIGS. 6–8.—Sections through developing tympanic membranes which occurred in connection with the various types of operations performed. The various layers of the integument correspond to those labeled in Plate I.

FIG. 6.—Membrane formed in back-skin graft transplanted to tympanic membrane region. The central portion is not as completely developed as the peripheral areas. (Case ABT 2.) ×110.

FIG. 7.—Membrane developing in a side-skin graft transplanted to tympanic membrane region. (Case APT 11.) ×110.

FIG. 8.—Membrane formation in side skin underneath which an annular tympanic cartilage transplant had been placed. The cytological changes are most marked in the skin directly over the rim of the cartilage transplant. *CT*, portion of annular tympanic cartilage transplant. (Case ATC 19.) ×110.

PLATE II



phosis, providing, of course, the grafts so made, were well nourished in their foreign location and acquired a good blood supply. If, however, the latter alternative existed, we should expect that isolation of the integument from the developing structures of the middle ear would result in the failure of tympanic membrane formation in such grafts during metamorphosis. Reciprocal grafts would also tend to throw light on this question, in that integument of the back and side would be expected to transform normally when transplanted to the tympanic membrane region if structures of the middle ear were responsible for tympanic membrane formation. Conversely, if only the integument of the tympanic membrane region is capable of tympanic membrane formation, we should not expect foreign integument to transform likewise when transplanted to that area.

2. TECHNIQUE OF SKIN TRANSPLANTATIONS

The following technique was employed in making all skin transplantations: The anesthetized animal was placed on a damp cloth, and the area of skin to be removed was carefully cut by means of a finely sharpened pair of iridectomy scissors. Considerable care must be exercised in this regard, so as not to cut the thin peritoneum beneath. The square of skin so outlined is then grasped with a fine-pointed forceps and slowly pulled off, the connecting strands of subcutaneous connective tissue being cut with a small spear-head scalpel. Where reciprocal grafting was employed, both grafts were first freed from the peritoneum, after which they were interchanged. The only precaution necessary when adjusting the grafts is in carefully flattening out the edges, which have a tendency to curl up beneath and so retard proper adhesion.

In consequence of the tendency of skin grafts to shrink in size when removed, a marked wound area surrounding the grafts is obtained upon transplantation. In general, this necessitated the transplantation of a graft to the tympanic membrane region somewhat larger than that of the reciprocal transplant removed from that area, since adhesion and healing progress more slowly at this point than in the case of transplants placed on the back or side. It was therefore possible to fit the transplanted graft of skin quite accurately to the denuded area of the tympanic membrane region.

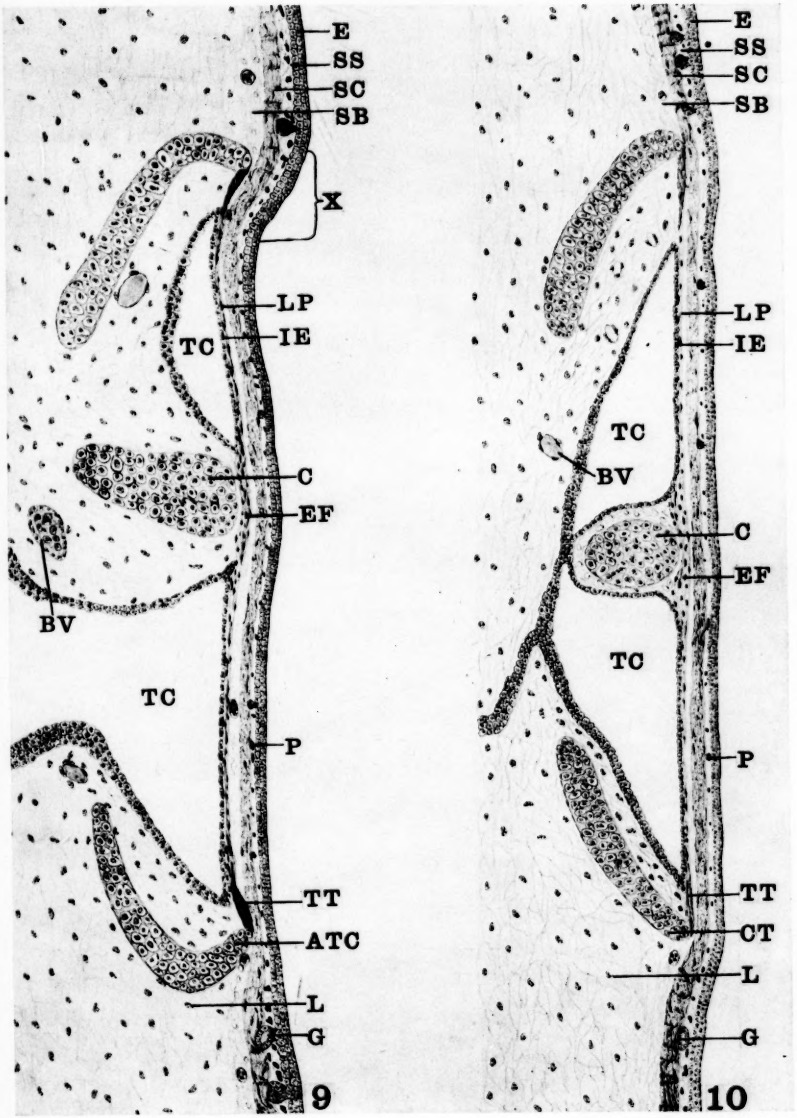
PLATE III

FIGS. 9, 10.—*E*, epidermis; *SS*, stratum spongiosum; *SC*, stratum compactum; *SB*, subcutaneous connective tissue; *LP*, lamina propria; *IE*, inner epithelial layer; *P*, pigment masses; *C*, tip of columella; *EF*, thickened elastic fiber region of lamina propria; *TT*, tensor tympani; *TC*, tympanic cavity; *ATC*, normal annular tympanic cartilage; *CT*, annular tympanic cartilage transplant; *G*, skin glands; *L*, lymphocytes; *BV*, blood vessels; *X*, transition region between tympanic membrane and surrounding integument.

FIG. 9.—Cross-section through a normal tympanic membrane, showing its associations with the columella and annular tympanic cartilage. ×30.

FIG. 10.—Cross-section through a tympanic membrane formed in the skin of the back under which an annular tympanic cartilage transplant had been grafted. The section includes the transplant, columella, and adjacent tissues. (Case CB 27.) ×40.

PLATE III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This, obviously, resulted in relatively large wound areas surrounding the grafts placed on the back and side.

Following the adjustment of the grafts, the anesthetized animals were placed in a small amount of oxygenated water for recovery. The animals were so placed that the operated areas were exposed to the air, which condition tended to hasten the congealing of the lymph and blood which serve to hold the graft in place until definite

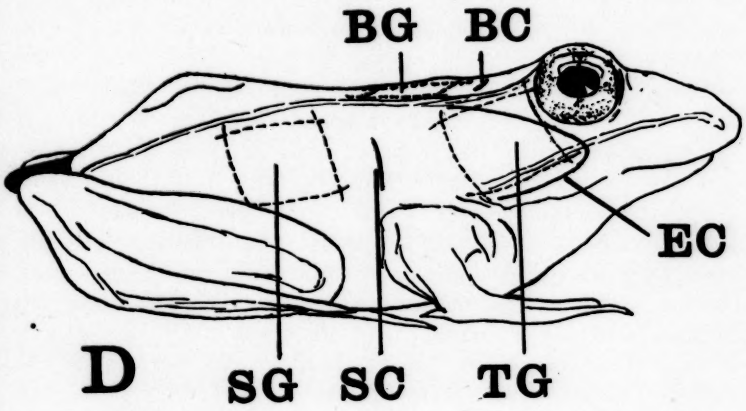


FIG. D

healing has occurred. Upon recovery, the animals were immersed in water, fed *Spirogyra*, and allowed to metamorphose.

3. SERIES APT

This series consisted of the reciprocal transplantation between skin grafts of the side and tympanic membrane region (*SG*, *TG*, Fig. D). The metamorphic stage operated on varied considerably and covered a range from the stage immediately following fore-limb eruption to one near the end of metamorphosis when the tail had been resorbed to a small black stump. The grafts taken from the tympanic membrane regions of the tadpoles in the earlier stages of transformation were in very early histological stages of tympanic membrane formation. Those taken from later stages were considerably more advanced, histologically, and presented fairly distinct pictures, macroscopically, of tympanic membrane formation. The

side grafts transplanted to the tympanic membrane region, however, represented normal integument in all cases.

Within three days following transplantation, the grafts were usually fully healed, the fusion area being represented by a narrow black line. The various steps taken during this healing process have been described by the writer in a previous paper (1926) and will not be described here. The reader is also referred, in this connection, to the works of Peters (1885), Barfurth (1891), Loeb (1898, 1902), Harrison (1910, 1914), Holmes (1913, 1914), Oppel (1913), Cole (1922), *et al.*

Following the healing period, no definite visible changes were observed for from 3 to 5 days, during which time the tadpoles were rapidly completing the last stages of metamorphosis. At that time, however, distinct pigmentary changes occurred in the grafts transplanted to the tympanic membrane region, which were typical of normal tympanic membrane formation. These were shortly followed by a perceptible outlining of the area exhibiting pigmentary changes. The area so outlined was similar in shape, size, and orientation to that of a normal tympanic membrane. In a few cases more or less translucent, crescent-shaped areas formed at the periphery of the areas, usually on the ventral boundary. Further modifications of these areas consisted chiefly of a gradual change in pigmentation, during which a brownish coloration was assumed similar to that possessed by normal tympanic membranes. The areas also presented slight indentation in general. Failure of the animals to live longer than 10–14 days following the operations, however, prevented the further development of tympanic membrane characteristics. The characteristic appearance of the grafts just prior to the death of the animals is represented in Figures 17 and 18, Plate V.

Several of the grafts were sectioned and examined, microscopically, for possible histological signs of tympanic membrane formation. In all cases where external changes had been recorded, definite transformations had occurred which were typical of normal histological membrane development (Fig. 7, Plate II). It was noted in particular that the portions of the graft which had been in contact with the rim of the annular tympanic cartilage were especially well

advanced in this transformation. The stratum spongiosum at such points was obliterated, while the stratum compactum presented the typical granular and fibrous condition as found in the fully developed tympanic membrane. The presence of lymphocytes in considerable abundance and the dissociated appearance of the stratum compactum of the central regions further emphasized the conclusion that definite histological transformations, identical with those of normal tympanic membrane formation, were in operation.

The fate of the reciprocal, autoplastic grafts transplanted from the tympanic membrane region to the side presented little or no external modifications. Following the healing period, the only marked visible tendency was to assume, in general, the same depth of pigmentation as presented by the surrounding integument. Histological sections of such grafts showed quite clearly that the various layers of the integument were rapidly assuming a normal structure. Grafts that had been transplanted when in early stages of tympanic membrane formation presented the histological appearance of normal integument. In other words, the layers of the integument which had undergone slight histological changes shortly retrogressed to the condition of normal integument upon transplantation. In the case of grafts transplanted during later stages of tympanic membrane formation, the retrogression was not so complete, since more pronounced changes had taken place prior to transplantation. In general, it was clear that such integument never tended to complete the histological tympanic membrane changes present when removed from the tympanic membrane region, but on the contrary, always underwent transformations directed to establish the normal integumentary picture.

4. SERIES ABT

Series ABT animals involved the same type of operation as employed for Series APT individuals, except that grafts of skin from the back were interchanged with those of the tympanic membrane region (*BG*, *TG*, Fig. D). The individuals used were generally in somewhat later stages of metamorphosis than those of the preceding series, since the best results of the latter group had been obtained with such forms. The average specimens employed were nearing the end of metamorphosis, the tail being reduced to a stump, and the

tympanic membranes appearing quite distinct as to shape and texture. The operation, therefore, really consisted of removing a square of skin containing the half-formed normal tympanic membrane and interchanging it with a square of normal integument of the back.

The back-skin grafts, so transplanted, were deeply pigmented and presented a uniform black coloration. Within 4 days, on the average, the grafts were fully healed and assumed a much lighter, brownish shade. In a few cases the earliest signs of tympanic membrane formation appeared on the sixth day following transplantation to the tympanic membrane region. The average time required for all cases was about 9 days, however. Following this, the usual macroscopic developments of tympanic membrane formation occurred in logical order. It was at once apparent that development was proceeding to a far greater degree in these individuals than in those of the earlier series. This will be evident by reference to Figure 13, Plate IV, which represents the typical condition of the developing tympanic membrane when death occurred on the twelfth to fifteenth day following transplantation. The tympanic membranes, so formed, were similar in all respects to normal membranes as developed on the opposite sides of the same individuals (Fig. 11, Plate IV). In general, it was noted, however, that the degree of development was always somewhat behind that of the normal left-side membranes—a condition to be expected in view of the comparatively shorter period of time available for right-side membrane development.

Microscopic examination of the membranes so developed revealed, as in the preceding series, the typical cellular modifications of normal membrane formation. The extent of the histological transformations, however, was in all cases considerably more advanced and consistent with the degree of development as evidenced by the external stage of membrane formation. Figure 6, Plate II, represents the typical condition found. It will be noticed here that all but a small central area of the membrane presents the cytological conditions typical of a fully formed membrane. Evidently this is the last portion of the membrane to complete its development. The reappearance of the stratum spongiosum with scattered glands and

the relative scarcity of lymphocytes further emphasize the late stage of membrane formation present.

The reciprocal, tympanic membrane grafts, transplanted to the back, underwent similar retrogressive transformations as already described for grafts transplanted to the side. Externally, the grafts tended to assume the darker pigmentation of back skin while the half-formed tympanic membranes became less conspicuous and finally lost their characteristic, brownish coloration and texture. The area could always be identified, however, due to the persistence of a certain amount of indentation. Histologically, the grafts again presented definite signs of retrogression and a decided transformation back toward the conditions typical of normal integument.

ANNULAR TYMPANIC CARTILAGE TRANSPLANTATIONS

1. PURPOSE OF THE CARTILAGE TRANSPLANTATIONS

The results of the reciprocal grafting of back and side skin with skin of the tympanic membrane region showed quite clearly that the skin of the latter is not peculiarly, hereditarily specific toward tympanic membrane formation. When transplanted to foreign locations, it fails to develop membranes during metamorphosis; while at the same time back and side skin transplanted to the tympanic membrane region transform into normal tympanic membranes. Such results lead one, therefore, to the conclusion that structures peculiar to the tympanic membrane region must be responsible in producing the transformations induced in the integument of that region during metamorphosis.

The most obvious structure in this region, and the only one in contact with the integument, is that of the annular tympanic cartilage. The possibility that it exercises a developmental influence on the formation of the tympanic membrane is strengthened when we consider its parallel development with that of the latter. It is always the skin directly over the cartilage which transforms into membrane. Moreover, the size, shape, and orientation of the membrane is identical with that of the annular tympanic cartilage.

Obviously, the extirpation and transplantation of the cartilage at various stages of development should throw light on this ques-

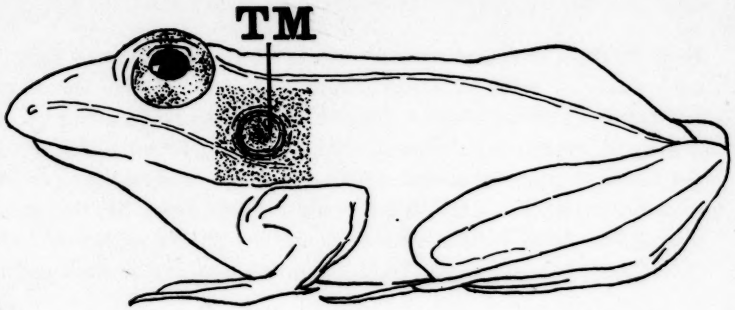
PLATE IV

FIG. 11.—Normal control tympanic membrane as developed on the left (unoperated) side of individuals whose right annular tympanic cartilage had been extirpated. *TM*, tympanic membrane. ×4.

FIG. 12.—Sketch showing absence of tympanic membrane formation during metamorphosis following previous extirpation of the annular tympanic cartilage. *EC*, scar of extirpation cut made when removing annular tympanic cartilage. ×4.

FIG. 13.—Formation of a tympanic membrane in a back-skin graft previously transplanted to the tympanic membrane region. *BG*, back-skin graft; *TG*, skin graft from tympanic membrane region transplanted to the back; *TM*, tympanic membrane. (Case ABT 30.) ×4.

PLATE IV



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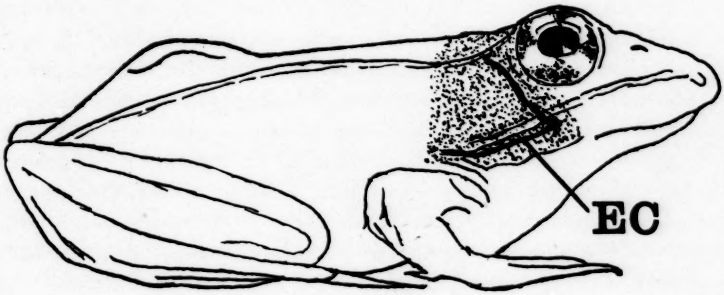

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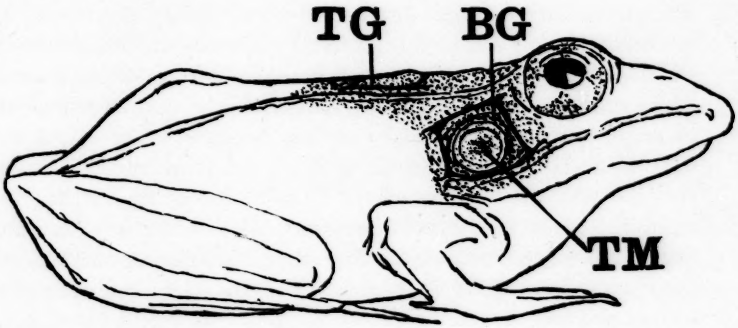

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tion. If the cartilage normally exerts such an influence, then its extirpation at an early stage should inhibit the development of a tympanic membrane, either partially or wholly, depending on the degree of influence. Furthermore, should tympanic membranes fail to develop in the absence of the cartilage, it should be possible to form tympanic membranes in any integument by the transplantation of the cartilage beneath and in contact with such skin. Operations were consequently designed to investigate such points.

2. TECHNIQUE OF CARTILAGE EXTIRPATION AND TRANSPLANTATION

The technique employed in extirpating the annular tympanic cartilage was subject to some variation depending on the stage during which the cartilage was removed. It will be remembered in this regard that the developing ring of cartilage undergoes a gradual migration anterioposteriorly during metamorphosis. In general, however, the following method was employed: The tadpoles were anesthetized, and a flap of skin (*EC* Fig. D) was carefully cut and laid back in order to expose the developing cartilage. In this regard it was necessary to avoid cutting the skin at any point destined to transform into tympanic membrane during normal metamorphosis. The ring of cartilage was then carefully separated at the periphery from adjoining tissues and severed from its attachment by snipping with a fine-pointed pair of scissors. By using considerable care, it was possible in this way to remove the ring of cartilage at any stage of development with a minimum of bleeding. The prevention of excessive bleeding is very important in metamorphosing tadpoles, since they are generally quite weak during this period and fail to recover following loss of much blood. Following the removal of the cartilage, the flap of skin was then carefully replaced and the cut edges smoothed out to facilitate healing. The next step consisted in transplanting the ring of cartilage beneath either side-skin or back-skin. This was accomplished in the following manner: A small cut just sufficient to allow of the introduction of the cartilage was made in the skin at a point considerably anterior to the desired orientation of the transplant. The extirpated ring of cartilage was then pushed through this opening by means of a small spear-head scalpel and forced back under the skin. In this regard it was neces-

sary first carefully to loosen the skin from the underlying peritoneum of the side or muscles of the back, as the case might be. Care was also exercised to have the lateral side of the cartilaginous ring facing and in contact with the skin, in order to duplicate the normal orientation as found in the tympanic membrane region. The operated animals were then placed on their sides in shallow water, to allow adhesion of the flap of skin over the extirpation area, and allowed to recover from the anesthetic.

3. SERIES ATC

This series consisted of the extirpation of the annular tympanic cartilage in various stages of development and its autoplastic transplantation beneath the skin of the side. The stage operated on varied from one in which the tail was but half-atrophied and only the earliest external signs of tympanic membrane were visible, to one near the close of metamorphosis when the tail was reduced to a mere stump and the tympanic membrane was about half-formed. The annular tympanic cartilages of the early stages were quite small, at which time they are found developing ventrally and somewhat anteriorly to the eye (*ATC*, Fig. A). Those removed from animals in the later stages of metamorphosis were comparatively much larger and circular in shape and occupied a more posterior location (*ATC*, Fig. B). Transplantations of the annular tympanic cartilage were made at all intervening stages between these two extremes.

The effect on the skin of the side in the region of the cartilage transplants varied considerably according to the stage of the annular tympanic cartilage transplanted. In the case of the early-stage transplants, only slight or no external change was observable. The integument generally appeared quite normal and gave no evidence, either through coloration or bulging, of the presence of the cartilage. Subsequent examinations of such individuals showed that the transplants in most cases had failed to thrive in their new location, and, while a few were attached to the under side of the integument, practically none had established connections with the peritoneum. The small size of such transplants, furthermore, made strong contact with the integument impossible. It was quite evi-

dent that a good proportion of the transplants had undergone slight disintegration, and in no case had growth taken place. Sections made through the integument in contact with the cartilage gave little or no evidence of tympanic membrane changes.

Pronounced macroscopic and microscopic changes were found to occur in the side skin where the larger and later-stage transplants were employed. Here, a distinct though slight bulging of the skin occurred, due to pressure of the transplant beneath; and the area not infrequently assumed a light-brownish coloration, indicative of early tympanic membrane formation. Distinct outlining occurred in several cases, which coincided with the rim of the cartilage transplants beneath. The outlining was evidenced by a pronounced grayish translucency of the skin (*ATC*, Figs. 15 and 16, Plate V) and was present only when, as subsequent examinations showed, the integument was in strong contact with the transplant.

Histological sections made through such integument showed that typical histological tympanic membrane transformations were well under way (Fig. 8, Plate II). It was also clear from the sections that the degree of tympanic membrane formation was in direct proportion to the degree of contact of the skin with the transplants. It was furthermore noted that the time element played an important rôle, in that individuals surviving for longer periods of time presented the most pronounced signs of tympanic membrane formation. The logical explanation for this is that the transplants in such cases had a longer time in which to influence the development of cytological changes in the integument.

The extirpation of the annular tympanic cartilage prevented the development of tympanic membranes in the normal location in all cases. When the transplants had been removed during an early stage of development, the integument of the tympanic membrane region remained normal throughout metamorphosis and resembled in all respects, both visibly and histologically, normal skin of the neck (Fig. 12, Plate IV). When the transplant had been extirpated at a late stage of development and not until the normal tympanic membrane had been half-formed, there was a decided tendency for the membranes to retrogress and assume the external and microscopic characteristics of normal integument. In either case, the

results indicated quite conclusively that the annular tympanic cartilage was essential for membrane development. In this regard it may be said that the tympanic membranes as developed on the left and unoperated side (Fig. 11, Plate IV) were always well formed and served as controls for all extirpations made on the right side.

4. SERIES CB

Series CB operations resembled the transplantations as described above in all respects except that the transplants were made beneath the skin of the back instead of beneath side skin. The transplants when extirpated varied as regards their degree of development, although the majority were in late stages of formation, many having attained the size indicated in Figure B, *ATC*. Because of their relatively large size, they were always in strong contact with the integument when transplanted, and produced a strong bulging of the latter.

The first macroscopic signs of tympanic membrane formation occurred within 4–5 days following transplantation. Unfortunately, however, owing to the late metamorphic stage of the individuals operated on, no cases survived longer than 5 days following this period. The time available, however, was sufficient for the formation of several remarkably well developed membranes, which were identical in all respects to normal tympanic membranes (*TM*, Fig. 14, Plate V). The development in certain cases was so perfect that the finer, characteristic color shadings and peculiar texture of the membrane were fully developed.

Several serial sections were made through such membranes, the sectioning including the cartilage transplants beneath (Fig. 10, Plate III). Such sections revealed remarkably identical histological pictures when compared to similar sections made through normal tympanic membranes of the head region, as pictured in Fig. 9, Plate III. The similarity not only included the various layers of the integumentary portion of the tympanic membrane but also the lamina propria and internal epithelial layers. It was also evident that the cartilage transplants were in a healthy condition and their cells undergoing normal division. The presence of a supporting, loose connective tissue containing scattered lymphocytes and other

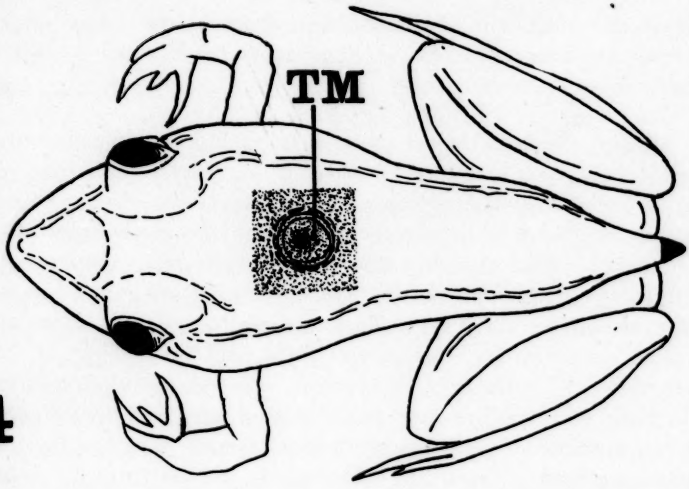
PLATE V

FIG. 14.—Formation of a tympanic membrane in skin of the back underneath which an annular tympanic cartilage had previously been transplanted. *TM*, tympanic membrane. (Case CB 15.) ×4.

FIGS. 15, 16.—Partial tympanic membranes formed in skin of the side underneath which annular tympanic cartilages had previously been transplanted. *TM*, tympanic membrane; *ATC*, outlining of membrane, indicating position of annular tympanic cartilage transplants. (Cases ATC 29 and 19, respectively.) ×6.

FIGS. 17, 18.—Partial tympanic membranes formed in side-skin grafts previously transplanted to the tympanic membrane region. *SG*, side-skin grafts; *ATC*, translucent areas indicating presence of annular tympanic cartilage beneath skin; *O*, outlining of developing tympanic membrane indicating location of annular tympanic cartilage. (Cases APT 6 and 15, respectively.) ×6.

PLATE V



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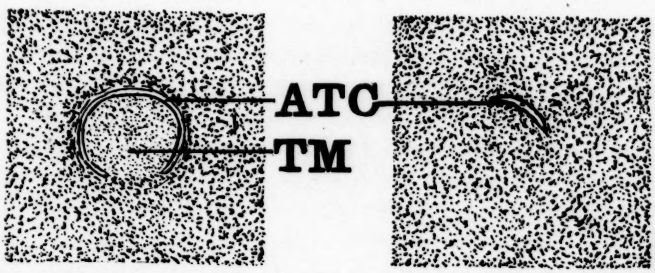

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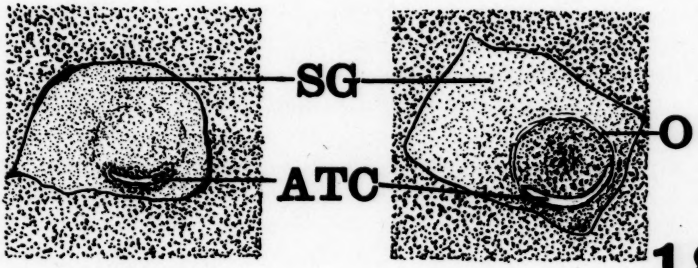

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blood cells completed a picture indistinguishable from that of the normal membrane and adjacent structures.

DISCUSSION

The formation of the tympanic membrane presents many histological structures of interest, especially as regards their morphological and embryological nature. The replacement of the stratum compactum by a fibrous layer is perhaps the most noteworthy histological transformation. That this layer is really structurally different from the compactum layer of normal integument seems quite evident to the writer. That two distinct sets of fibers are present is also evident and in agreement with the findings of Tokura (1925). The mode of their origin, however, is quite problematic. The apparent breaking up of the normal compactum layer and its invasion by lymphocytes would suggest that the layer is completely broken down and removed. If this be true, the fibers of the new compactum layer of the future tympanic membrane must be formed from other connective tissue elements which make their way to this region at this time. The fact, however, that the formation of the fibers and the breaking down of the old compactum layer appear to occur simultaneously during the late stages of the disruption of the layer would suggest that the fibers may possibly be formed from dissociated elements. Certainly it is true that by the time phagocytic action is on the wane, the fibers with their characteristic granulated appearance can be observed and identified from the dissociated remnants of the compactum layer. Furthermore, the gradual merging of this fibrous layer with the stratum compactum of the surrounding integument would also suggest such a developmental transformation.

The results of the skin-transplantation experiments (Series APT and ABT) have emphasized the fact that the integument of the tympanic membrane region is not a self-differentiating tissue as regards its ability to transform into tympanic membrane during metamorphosis. It is apparent that in this physiological respect, at least, all integument possesses like potentialities, in that foreign skin is capable of tympanic membrane formation when in the tympanic membrane region. This same tendency of integument to

react alike, physiologically, has also been demonstrated by the writer (1926) in connection with the formation of the opercular fore-leg perforations. It was found in this connection that integument of the back and side, when transplanted to the region of fore-limb eruption, underwent typical histolysis which resulted finally in the appearance of a normal perforation. In the present problem, the fact that integument of the tympanic membrane region when transplanted to the back or side failed to develop into tympanic membrane during metamorphosis, further emphasizes the conclusion that such integument is not hereditarily specific for membrane formation, but undergoes such transformations owing solely to its association with structures of the middle ear.

The experiments involving extirpation and transplantation of the annular tympanic cartilage indicate quite conclusively that this structure constitutes the immediate influence responsible for the development of the tympanic membrane. It is also quite evident that it is the only causal agent concerned, since removal of the cartilage inhibited the development of even the earliest signs of membrane formation. Conversely, the membranes formed in foreign integument under which the cartilage had been transplanted, were typical in all respects, including the specific histological developments and the formation finally of well-formed membranes.

The period of development of the annular tympanic cartilage during which it is physiologically active in membrane formation is an important point. From the results of the cartilage-transplantation experiments, it would appear that it possesses such an influence from a very early stage of development until the stage reached at the close of metamorphosis. This physiological influence, although very slight at first, gradually increases, and reaches a maximum slightly before metamorphosis is completed. It is interesting to note here, that although the cartilage is physiologically active at a very early developmental stage, it apparently exercises no influence toward the formation of the normal tympanic membrane at that time. This is undoubtedly due to the fact that the cartilage is not in contact with the integument at the time but is undergoing a posterior migration. It is only when the cartilage reaches the location typical of membrane formation that it is brought into firm

contact with the integument, to which it shortly adheres. It is evident, then, that although the cartilage is physiologically active as regards membrane formation at a very early developmental stage, it cannot exercise this power until its migratory development has brought it in strong contact with the integument.

The fact that actual contact of the cartilage with the integument is essential before histological tympanic membrane changes may occur in the latter, naturally raises the question regarding the mode of influence in operation. It is just possible that the pressure exerted against the integument by the cartilage is sufficient partially to occlude the blood supply to the former. This, in time, would produce a condition of acidosis resulting from the failure of the blood stream adequately to remove products of excretion by the tissue, and in this manner initiate the histolytic processes typical of the early stages of membrane formation. It is difficult to see, however, how a condition of acidosis capable of producing histolysis could be responsible for the formative transformations involved during the later stages of membrane formation. A more logical assumption, according to the writer, would be to assume the action of a specific secretion or hormone of the annular tympanic cartilage. Such a secretion or hormone, applied by direct contact to the integument, could function as the causative agent of histolysis at first, and later as a stimulus to the development of the structural components of the adult membrane. It is also quite logical to assume that two hormones may be concerned—one responsible for the early histolytical changes, and the other for the later formative developments.

Regardless of the exact nature of the physiological influence exerted, it is clear that the influence not only requires direct contact with the skin to become active but requires a considerable period of time to bring about the development of a complete tympanic membrane. If, for example, the annular tympanic cartilage is extirpated at a time when the membrane is forming, the result is to inhibit all further tympanic membrane developments. In fact, there is a decided tendency for the incompletely formed structures to undergo histological changes designed to re-establish the structure of normal integument. This was evident not only in many cases of cartilage extirpation but also in all cases where developing

membranes were transplanted to the back or side and therefore away from the influence of the cartilage. The fact that considerable enlargement of the tympanic membrane takes place following metamorphosis, during the growth of the young frog, would suggest, strongly, that the annular tympanic cartilage continues to exert its influence on membrane formation during this period also.

The mechanism of tympanic membrane formation, finally, serves as a good example of what might be called the "indirect action" of the thyroid hormone in amphibian metamorphosis. It is the thyroid hormone which primarily sets in operation certain fundamental reactions, which in turn bring about the development, transformation, or degeneration of other structures which in themselves may then effect the development or destruction of still other structures. Thus it is possible for the thyroid hormone to set in motion a series of interrelated reactions which finally bring about an end result. Some of the reactions concerned in the formation of the tympanic membrane of the frog would therefore appear to be as follows: The thyroid hormone either directly or indirectly initiates influences which bring about certain transformations of the skeletal system during metamorphosis. One of these transformations results in the development of the annular tympanic cartilage from the metapterygoid region of the quadrate. The annular tympanic cartilage during its growth generates an influence, probably hormonic in nature, which, when applied by contact to integument, initiates histological transformations resulting in the formation of a tympanic membrane.

GENERAL SUMMARY AND CONCLUSIONS

1. The formation of the tympanic membrane in *R. pipiens* is the result, histologically, of a definite, orderly process involving the histolysis and reconstruction of certain integumentary layers. The appearance of numerous, invading lymphocytes is a secondary and phagocytic phenomenon and is not to be regarded as causing the histolysis.

2. Autoplastic skin grafts from the back and side were interchanged with skin grafts from the tympanic membrane region, and the tadpoles were allowed to metamorphose. Typical tympanic

membranes formed in the grafts transplanted to the tympanic membrane region. The integument of the tympanic membrane region, when transplanted to the back or side, failed to develop membranes.

3. The results of skin transplantation indicate that all integument is capable of transforming into tympanic membrane when in the tympanic membrane region. The normal integument of that area is not, therefore, a self-differentiating and hereditarily specific tissue.

4. The annular tympanic cartilage was extirpated at various stages of development, and the individuals metamorphosed. No cases of membrane formation occurred following extirpation of cartilages in early developmental stages. Extirpation of cartilages, when the membrane had begun to form, prevented further development of membranes. The membranes subsequently tended to transform back into normal integument.

5. Annular tympanic cartilages were transplanted, autoplastically, in various stages of development beneath the skin of the side and back. Tympanic membranes were formed in such locations directly over the transplants. The completeness of the membranes formed was dependent on the developmental stage of the cartilage transplanted, the length of time the skin was in contact with the transplant, and the degree of contact existing between transplant and skin.

6. The conclusion is reached that the histological transformations in the integument during tympanic membrane formation are the result of influences exerted by the developing annular tympanic cartilage. The suggested influence is that of a secretion or hormone applied by direct contact to the integument by the cartilage.

7. The mechanism of tympanic membrane formation illustrates an indirect method of the thyroid hormone in bringing about certain metamorphic transformations. In this case it would appear that the thyroid hormone either directly or indirectly stimulates the metapterygoid region of the quadrate to form the annular tympanic cartilage, which, as development progresses, liberates an influence, probably hormonic in character, whose application to the integument induces the histological transformations essential for tympanic membrane formation.

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THE RELIEF OF PARATHYROID TETANY BY INJECTIONS OF URANIUM NITRATE

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RECENT experimental studies have indicated that slight changes in the acid-base equilibrium, with a shift in the reaction of the blood toward the acid side, exerts a profound effect upon the tetany of parathyroid insufficiency. For example, Wilson, Stearns, and Janney (1915) report that injection of 0.1–0.5 gram of hydrochloric acid promptly brings relief to dogs suffering from severe tetany. The work of these investigators was followed by that of György (1922), Gamble and Ross (1923), and Anderson and Graham (1924), who were able to demonstrate that acid-producing salts such as calcium chloride and ammonium chloride are highly effective agents in the treatment of infantile tetany and, when given in sufficient quantity, relieve all symptoms.

Boyd, Austin, and Ducey (1926), and Wenner (1927) working independently, repeated the experiments of György, and Gamble and Ross, using parathyroidectomized dogs as experimental animals. It will be recalled that parathyroid removal when practiced on dogs is almost invariably fatal, and the tetany symptoms are far more severe than those usually observed in infants. However, ammonium-chloride administration brings about a prompt cessation of symptoms, even the most violent, within a short time, and the animals return to an apparently normal state. The serum calcium of dogs so treated generally, though not invariably, shows a slight increase, but never rises to the preoperative level normal for this species, i e., 10–11 mgm. per 100 cc. of blood.

These observations indicate that the administration of NH_4Cl, by increasing HCl metabolism and decreasing the pH of the blood, somehow influences the solublity of calcium, thereby increasing the amount of this substance in the circulating fluids of the body and bringing about relief from tetany. It is fast becoming obvious that it is not the quantity of total calcium in the blood and tissues which

is important in tetany prevention and relief, but the solubility of certain types of calcium compounds. This point will be more fully discussed later.

Swingle, Wenner, and Stanley (1928) demonstrated that partial asphyxiation of parathyroidectomized dogs in violent tetany by CO_2 promptly relieves symptoms. The animals return to normal and remain free from symptoms for some hours. Dogs treated with CO_2 exhibit marked acid intoxication, as shown by lowered CO_2 capacity, CO_2 content, and pH of the blood. It was demonstrated that the acidosis was not due to CO_2 but to rise in lactic acid due to anoxemia. When the blood lactic acid returned to the normal level (approximately 28 mgm. per 100 cc.), tetany recurred. Despite the fact that the CO_2-treated dogs appeared free from tetany, the serum calcium remained low and was not markedly affected by the CO_2 treatment. A slight rise in calcium invariably occurred, but in several instances was within range of the experimental error of the method employed for its determination. It is our opinion, however, that the relief from tetany which followed the use of CO_2 was due to the increased solubility of certain types of calcium compounds at the lowered pH.

Greenwald and Gross (1925 *a*, *b*) observed, in their study of the pathogenesis of tetany, that following parathyroidectomy there is no increased excretion of calcium, as reported by Salvesen (1923), but an actual decrease in the elimination of both calcium and phosphorus. Whereas, after injection of Collip's active parathyroid extract, increased excretion of both calcium and phosphorus occurred. They suggest, on the basis of their observations, that after parathyroidectomy, calcium is precipitated in the tissues as calcium phosphate, which in the absence of the parathyroid hormone becomes less soluble. In the light of their observations, it seemed likely that a slight shift in the reaction of the blood toward the acid side increases the solubility of certain calcium compounds, and that any substance inducing such a change in reaction would prove effective in the treatment of parathyroid tetany.

During the course of certain acid-intoxication experiments on dogs which involved the use of nephrotoxic substances such as uranium nitrate, the idea occurred that this drug should be useful

in controlling and relieving tetany because of the marked change in the reaction of the blood toward the acid side which follows its administration. It has been known for years that following subcutaneous injections of uranium nitrate, a marked acid intoxication slowly develops which is likely to prove fatal if large doses of the drug are used. The work of MacNider (1920) and others showed that uranium nitrate is a powerful nephrotoxic drug and induces severe degenerative changes in the tubular epithelium of the kidneys. The nature of the acid or acids which are responsible for the acidosis following its use is not known for certain.

The idea which most readily comes to mind with regard to the cause of the acidosis of uranium-nitrate poisoning is that since this substance is nephrotoxic and causes kidney lesions, the acid-eliminating functions of the kidneys are interfered with to such an extent that acid substances formed in the course of metabolism and normally eliminated by the kidneys are retained. These substances accumulate in the blood and eventually induce the type of acid intoxication characteristic of the terminal stages of uremia. Although the supposition seems logical enough and is commonly accepted as an adequate explanation, it nevertheless does not fit the facts when subjected to scrutiny, for the following reasons:

1. According to MacNider, who has worked extensively with uranium nitrate, the acidosis which develops following its injection into dogs may appear before degenerative changes in the kidneys become evident.

2. Atchley and Benedict (1927) and Swingle, Wenner, and Stanley (1928) have demonstrated that following bilateral ureter ligation and total kidney extirpation, acid intoxication, if present at all, in such operated animals is not very marked.

3. Wallace and Pellini (1921) report the case of a dog from which both kidneys had been removed, which showed no acidosis. However, when the animal was injected with uranium nitrate, severe acid intoxication developed.

In the experiments reported here, our chief interest was in the effect the acidosis of uranium-nitrate poisoning might have upon the relief of parathyroid tetany, but an attempt was also made to determine the nature of the acid or acids responsible for the intoxication itself.

Ten dogs were employed in the experiments. They were of various ages and sizes, complete data concerning some of them are given in the protocols. The animals were parathyroidectomized and allowed to develop marked tetany symptoms. They were then bled for CO_2 capacity, pH, and serum calcium. It has been the experience of those working in this laboratory, Wenner and Muntwyler (1927), and others, that the CO_2 capacity and pH of tetany dogs are normal; and our own personal experience corroborates this view, except when a dog has just recovered from a series of violent convulsions, for in such cases one finds a lowered blood pH and CO_2 capacity, due to rise in lactic acid. Omitting such exceptional cases however, the blood of tetany animals is normal in so far as any evidence of acidosis or alkalosis is concerned.

Following bleeding, the tetany animals were injected with uranium nitrate subcutaneously. The dosage depended upon the age of the animal, in so far as we were able to determine the age of our mongrel dogs, for, as MacNider has emphasized, old dogs are much more susceptible to uranium nitrate than are younger animals. However, none of the dogs received more than 6 mgm. per kilo.

The effect of uranium nitrate upon parathyroidectomized dogs presenting violent tetany symptoms is indeed striking, for within 12–24 hours after injection, all manifest tetany symptoms have disappeared and the animals run about, eat, play, and act in normal fashion. A dog may be prostrate with tetany, unable to walk or even stand erect, yet within a short time following injection, the same animal presents no signs of tetany, and indeed differs in no way from an unoperated dog. The normal condition may last a week, 10 days, or even 2 weeks, before tetany again develops. Meanwhile the animal has been receiving a heavy meat diet. When tetany again appears, another injection of uranium nitrate is necessary, following which the tetany symptoms disappear; but generally the second injection is followed within a few days by symptoms of uranium poisoning which soon leads to death. No attempt was made to keep the animals alive indefinitely by employing very small doses of the drug when tetany recurred, although it is probable that this could be done. Complete data on two of our ten experimental animals are given in the protocols.

PROTOCOL 1

Female fox terrier. Weight, 8 kilos. Bled from heart, February 21. CO_2 capacity, 45.4 vols. per cent. pH, 7.38. Serum Ca, 13 mgm. Thyroparathyroidectomized February 13, 10:00 A.M. February 27, 9:00 A.M., dog in tetany. Muscular twitching and jerkings; animal spastic and refuses food. Bled. CO_2 capacity, 45.5 vols. per cent. pH, 7.37. Serum Ca, 7 mgm. per 100 cc. After bleeding, dog given 40 mgm. uranium nitrate subcutaneously, or about 5 mgm. per kilo of body weight.

February 28. Tetany symptoms absent; dog seems normal. Eats and plays.

March 2. Animal normal. Bled. CO_2 capacity, 21.1. pH, 7.23. Ca, 7.5 mgm.

March 3, 4, 5, and 6. Animal remained normal. Evening of the 6th, the animal seems listless and eats little. Shows symptoms of uranium poisoning.

March 7. Bled. CO_2 capacity, 19.8 vols. per cent. pH, 7.22. Ca, 7.2. Animal shows symptoms of severe acidosis, but no tetany.

March 8. Animal died of uranium-nitrate poisoning.

PROTOCOL 2

Mongrel pup, male. Weight, 7.5 kilos. Operated February 6, 2:00 P.M.

February 10. Tetany present. Animal very spastic; marked tremors and jerking of limbs. Salivating and panting. Bled. CO_2 capacity, 38.9 vols. per cent. pH, 3.34. Serum Ca, 7 mgm. per 100 cc. Dog injected at 10:00 A.M. with 45 mgm. uranium nitrate, or about 6 mgm. per kilo of body weight. Five P.M. of the same day animal ate meat and presented no tetany symptoms.

February 10–18, dog remained normal. February 18, dog bled. CO_2 capacity, 37.3. pH, 7.36. Ca, 8.2 mgm.

February 19–21. Animal perfectly normal. Bled February 21. CO_2 capacity, 28.3 vols. per cent. pH, 7.34. Ca, 8 mgm.

February 21–27. Animal free from tetany. Bled. February 27. CO_2 capacity, 35.3 vols. per cent. pH. 7.34. Ca, 8.5 mgm.

February 28—March 1. Animal normal. March 1, 3:00 P.M. Slight tetany symptoms apparent. Bled. CO_2 capacity, 35.5. pH,

7.35. Ca, 7 mgm. Animal given 3 mgm. uranium nitrate per kilo body weight.

March 2. Dog sleepy and listless; no tetany. March 3–8. Animal lethargic and apparently suffering from uranium-nitrate poisoning. Tetany symptoms absent.

March 9. Dog died of uranium-nitrate poisoning.

Table I gives in abbreviated form the data obtained from six of our nine cases. The remaining three animals behaved similarily to uranium-nitrate treatment, but were not bled so frequently for study of the acid-base equilibrium at various stages of the experiment.

Analysis of the data in Table I reveals several interesting facts. It is quite evident that the injected animals develop a severe acid intoxication following injection of uranium nitrate; the CO_2 capacity and pH drop markedly; and in most of our cases death eventually occurred from uranium-nitrate poisoning. The intoxication develops rather slowly and appears to be much more severe in some animals than in others. This variation is in all probability due to differences in the ages of the animals, since MacNider (1920) has clearly shown that old dogs react much more violently to uranium nitrate than do young ones. In fact, doses of the drug that are lethal for old animals may cause but transitory symptoms of acid intoxication in younger dogs.

Another point of interest is the rise in serum calcium which follows injection of uranium nitrate. While the increase is small, it invariably occurs; and in two cases it amounted to more than a milligram of calcium per 100 cc. of blood. This was true of animals No. 1, 2, and 4. Recently the writer and his collaborators (1928) reported similar small increases in the serum calcium following CO_2 administration to tetany animals. Wenner (1927) likewise reported increases after large doses of NH_4Cl. In all cases the increase in serum calcium which was noted appeared to be correlated with the fall in CO_2 capacity and pH. A slight shift in the reaction of the blood toward the acid side not only relieves tetany but induces a slight rise in the total calcium of the serum. This increase is not lasting, for the calcium may again decrease to levels lower than that observed when the tetany symptoms were first noted. For an example of this, note the data concerning dog 7 in Table I.

TABLE I

EFFECT OF URANIUM NITRATE ON PARATHYROID TETANY

Dog	Operated	Tetany	Bled	CO_2 Cap. Vols. per Cent	pH	Ca Mgm. per 100 Cc.	Remarks
1.	Jan. 27	Jan. 28	Jan. 28	40.7	7.38	6.2	Tetany. Injected 10 mgm. (2 per kilo) uranium nitrate 10:00 A.M. Jan. 28. Normal; ate and drank.
			Jan. 30	36.8	7.37	7.2	Condition fair. No tetany.
			Feb. 3	36.4	7.35	7.8	Ate. Injected 6 mgm. (1.2 per kilo) uranium nitrate.
			Feb. 4	29.8	7.25	5.8	No tetany. Injected 6 mgm. (1.2 per kilo) uranium nitrate. Died.
2.	Jan. 27	Jan. 30	Jan. 30	39.7	7.37	6.6	Slight tetany. Injected 78 mgm. (6 per kilo) uranium nitrate 9:30 A.M.
			Jan. 31	34.2	7.35	6.9	Animal normal.
			Feb. 2	32.4	7.33	7.9	Animal normal.
			Feb. 14		7.30	7.2	Refused food. Injected 65 mgm. (5 per kilo) uranium nitrate. Uranium poisoning.
4.	Feb. 6	Feb. 10	Feb. 10	38.9	7.34	7	Tetany. Injected (6 mgm. per kilo) uranium nitrate 10:00 A.M. Feb. 10. Recovery after 24 hours. Heavy meat diet.
			Feb. 12	37.3	7.36	8.2	Seems normal.
			Feb. 20	28.3	7.34	8.0	Animal normal.
			Feb. 27	35.3	7.34	8.0	Condition excellent.
			Mar. 1	35.5	7.35	7.0	Tetany. Refused food. Given 3 mgm. uranium nitrate per kilo.
			Mar. 8	24.2	7.27	6.8	No tetany. Symptoms acidosis.
			Mar. 10				Died uranium-nitrate poisoning.
7.	Feb. 11	Feb. 13	Feb. 11	47.1	7.39	8.0	Tetany, given 5 mgm. uranium nitrate per kilo. Normal after 36 hours. Eats meat and runs about.
			Feb. 15	40.9	7.35	7.1	No tetany symptoms. Heavy meat diet.
			Feb. 18	32.4	7.39	5.8	No tetany. Heavy meat diet.
		Feb. 21	Feb. 20	36.8	7.39	5.0	Tetany. Refused food. Given 3 mgm. uranium nitrate per kilo.
			Feb. 22	30.2	7.25	7.0	No tetany. In coma when bled. Died uranium-nitrate poisoning.

TABLE I—*Continued*

Dog	Operated	Tetany	Bled	CO_2 Cap. Vols. per Cent	pH	Ca Mgm. per 100 Cc.	Remarks
8.	Feb. 20		Feb. 20	41.2	7.40	10.8	
		Feb. 25	Feb. 25	34.2	7.36	5.9	Tetany. Spasm; unconscious 10 min. after bleeding. Injected 40 mgm. (5 per kilo) uranium nitrate.
			Feb. 27	28.7	7.22	6.2	Tetany disappeared in 24 hours.
			Mar. 2	22.3	7.24	6.0	Uranium-nitrate-poisoning symptoms present, however.
9.			Feb. 21	45.4	7.38	13.0	
	Feb. 23	Feb. 27	Feb. 27	45.5	7.37	7.0	Tetany. Injected 40 mgm. (5 per kilo) uranium nitrate.
			Mar. 2	21.1	7.23	7.5	Normal condition. Meat diet.
			Mar. 7	19.8	7.22	7.2	Symptoms of uranium poisoning; no tetany.
			Mar. 8				Animal died.
10.	Mar. 12		Mar. 12	45.4	[illegible]	12.4	
		Mar. 17	Mar. 17	42.4	[illegible]	7.4	Tetany. Injected 40 mgm. (5 per kilo) uranium nitrate. Normal after 36 hours. Eats meat; runs about.
			Mar. 19	37.3	7.33	8.2	No tetany symptoms. Heavy meat diet.
			Mar. 24	15.7	7.18	8.0	Uranium-nitrate-poisoning symptoms present. No tetany. Died.

Despite the fact that the dogs showed no gross manifestations of tetany and appeared normal, nevertheless the serum calcium remained low, in several cases as low as 5 mgm. of calcium per 100 cc. This was earlier observed in CO_2-treated animals.

The complete explanation why slight shifts in the acid-base equilibrium, with a change in reaction of the blood toward the acid side, should promptly relieve all tetany symptoms and bring about small increases in the serum calcium is not forthcoming in the present state of our knowledge of the pathogenesis of tetany. However, a tentative hypothesis, based upon facts which have recently come to light, is perhaps permissible.

It has been known for some years that blood serum contains more calcium than an ordinary aqueous solution of the same pH and phosphate content could maintain in solution. This fact has

been experimentally demonstrated by Holt, La Mer, and Chown (1925), who showed that when serum containing solid $Ca_3(PO_4)_2$ is shaken for some time, the concentration of both calcium and phosphate in solution decreases. This observation led Holt, La Mer, and Chown to suggest that blood serum is a supersaturated solution of calcium phosphate, and they advanced the hypothesis that serum contains a substance which delays the precipitation of calcium which is absorbed from the intestines.

Greenwald and Gross (1925*a*, *b*) observed that following parathyroidectomy there is a greatly diminished excretion of phosphorus in the urine, and furthermore that the retention of phosphorus is not, regularly at any rate, accompanied by an increase in the inorganic phosphate of the plasma. In later experiments the same investigators found that the decreased calcium content of the serum which invariably follows parathyroid removal is not due to increased excretion of calcium. In fact after gland removal, calcium excretion is much diminished, so that retention of calcium and phosphorus occurs. However, if an active extract of the parathyroid (Collip's) is injected, a marked increase in excretion of both calcium and phosphorus takes place. As a result of these observations, Greenwald (1925) advanced the hypothesis that in the absence of the parathyroid hormone, calcium and phosphorus are precipitated in the tissues in the form of calcium phosphate, thus accounting for the retained calcium and phosphorus and the simultaneous fall in the level of the serum calcium. According to Greenwald then, the parathyroid hormone, or some substance to the preparation of which the parathyroid hormone is essential, maintains calcium in solution in the plasma in excess of the quantity possible in its absence.

It becomes evident, therefore, that the ratio of calcium to phosphorus in the circulating fluids of the body of parathyroidectomized animals is highly important, and that anything tending to increase the elimination of phosphorus will make more calcium available. It seems probable that the relief from tetany symptoms and the rise in serum calcium which follows slight changes in the reaction of the blood toward the acid side, such as, for example, an intoxication by CO_2 or uranium-nitrate administration, is due to removal of phosphate. We have not demonstrated such elimination of phos-

phate in our experimental animals but are now engaged in this experiment.

While the increased excretion of phosphorus following development of acid intoxication remains to be proved for parathyroidectomized dogs, nevertheless such increased excretion has been demonstrated in cases of infantile tetany treated with acids and acid-forming salts such as ammonium chloride. Thus Scheer, György, and Anderson and Graham report that increased phosphate elimination occurs in such cases. It seems very likely that the relief of tetany which follows injections of uranium nitrate is due to increased phosphate excretion, which results from the shift in the reaction of the blood to the acid side, thereby rendering more calcium available owing to its greater solubility at the increased hydrogen-ion concentration.

During the course of the experiments an effort was made to determine the nature of the acid or acids responsible for the intoxication which follows injections of uranium nitrate.

Some years ago (1923) Denis reported that in severe uranium nephritis in animals, the inorganic sulphate of the blood is increased. In a later paper (1927), the same author reports that in dogs poisoned with uranium nitrate, the concentration of the inorganic sulphates and of the neutral sulphur fraction of the blood directly depended upon the volume of urine excreted. In animals with diuresis whose kidneys had been severely injured, there was frequently found a large decrease in the inorganic and total sulphates. As the volume of urine diminished to subnormal values, the concentration of inorganic sulphates and neutral sulphur rose; and in some cases attained value from 100 to 300 per cent above the normal concentration.

We tested two of our uranium-injected dogs for pH, CO_2 capacity, chlorides, phosphorus, and sulphates. The results of the tests are given in the combined protocols and tables.

URANIUM-NITRATE INJECTED DOGS

DOG A

White collie, male. Weight, 14 kilos. Uranium nitrate injected subcutaneously, 98 mgm. (7 mgm. per kilo of body weight). Bled

from heart 6 days later. Dog beginning to show symptoms of uranium-nitrate poisoning.

Bled 5 days later. Dog very weak; refused food for 24 hours; stiff when walking.

Died 3 hours after bleeding.

	pH	CO_2 Cap. Vols. per Cent	Cl Mgm. per 100 Cc.	P Mgm. per 100 Cc.	S Mgm. per 100 Cc
Normal	7.36	49.5	350	5	6.6
Six days	7.26	36.5	320	12	17.6
Terminal (11 days)	7.03	15.5	240	13.5	42.4

DOG B

Brown hound, male. Weight, 25 kilos. Uranium nitrate injected subcutaneously, 175 mgm. (7 mgm. per kilo of body weight). Bled from heart 6 days later. Dog beginning to show symptoms of uranium-nitrate poisoning.

Bled 4 days later. Dog very weak; refused food.

Died 2 days later.

	pH	CO_2 Cap. Vols. per Cent	Cl Mgm. per 100 Cc.	P Mgm. per 100 Cc.	S Mgm. per 100 Cc
Normal	7.40	51	340	5	3.9
Six days	7.11	31.5	340	14.25	14.9
Terminal (10 days)	7.09	25.1	270	15	37.6

It is evident from the data presented that marked retention of phosphorus and sulphate occurs following injections of uranium nitrate and that the blood chlorides fall. It seems probable that the acid intoxication which invariably accompanies uranium-nitrate poisoning is due in large part to retention of inorganic phosphorus and sulphate. The fall in chloride is obviously a compensatory reaction on the part of the organism in an effort to maintain the acid-base equilibrium in the face of retention of the other acids. The volume of urine excreted by our injected dogs diminished rapidly once uranium-poisoning symptoms developed.

SUMMARY AND CONCLUSIONS

1. Injections of uranium nitrate into parathyroidectomized dogs exhibiting marked tetany relieves all symptoms, and the animals return to an apparently normal condition within a few hours.

2. Dogs so treated remain normal for a week or 10 days before tetany recurs, despite the heavy meat diet given. Death results from uranium-nitrate poisoning.

3. Injected dogs, although free from tetany and apparently normal, have a low serum calcium. The serum calcium rises slightly some hours after injections, but the increase is seldom above 1.5–2 mgm. per 100 cc. of blood.

4. Uranium-nitrate injections induce a marked acid intoxication, as shown by lowered pH, CO_2 capacity, and CO_2 content.

5. The acidosis is probably due to retention of inorganic phosphate and sulphate. Blood chlorides tend to fall as phosphate and sulphate increase.

6. The relief from tetany symptoms which occur following uranium-nitrate treatment is apparently due to the shift in the reaction of the blood to the acid side, thereby rendering more calcium available owing to its greater solubility at the increased hydrogen ion concentration and to increased phosphate excretion.

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STUDIES IN ANIMAL AGGREGATIONS: MASS PROTECTION FOR PLANARIA FROM ULTRAVIOLET RADIATION

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RECENT experience of a number of workers, in this laboratory and elsewhere, has established that, other conditions being equal, survival after exposure to adverse conditions is frequently related tc the number of animals present; and that under certain conditions and within limits, the more animals the greater the protection. Much of the literature dealing with this and related subjects has been reviewed and listed elsewhere (Allee, 1927*a*). Interpretations of the phenomena differ. Drzewina and Bohn (1926) conclude from their observations on the relation between mass of animals and survival from exposure to colloidal silver, that the animals secrete an autoprotective substance. Bresslau (1924), using methylene blue, and Allee and Schuett (1927), with colloidal silver, find that many, if not all, of the facts they have observed can be explained on the basis of adsorption or other fixation of the toxic material.

When toxicity is due to the absence of some material rather than to its presence, there is a better opportunity to test for the possible presence of some secretion or excretion that might have a protective effect. Such conditions may be studied when marine animals are exposed to fresh water or to hypotonic sea water. This experiment, first tried by Bohn and Drzewina (1920) with the marine flatworm *Convoluta*, has recently been repeated by Allee (1928), with sea water contamination carefully checked. Under these conditions, *Procerodes wheatlandi*, also a marine turbellarian of the intertidal and adtidal zone, shows markedly greater survival if transferred to tap water in which other *Procerodes* have lived, died, and disintegrated than if it is exposed to freshly drawn tap water.

In these experiments with *Procerodes*, due care was taken in washing the worms adequately before introducing them into the

experimental conditions. The greater survival in the worm-conditioned tap water was much more marked than that in fresh tap water, even when sufficient dilute sea water had been added to more than equalize the chloride content of the two.

The protective effect was shown by water in which the worms had been allowed to disintegrate, or by the filtrate from this water before and after boiling. It was also carried to new dishes of tap water by washed carcasses of dead worms and was given off by living worms which were removed from the tap water before they died of the exposure. Obviously the worms conditioned this water by giving off some substance or substances which served to protect other worms when they were isolated into it. The nature of the protection furnished is not yet clear; only the fact of its existence has been established. The protection may well be due to the leaching or exudation of electrolytes that have the chlorine proportion different from that found in sea water. Further experiments are planned at this point.

Another type of experimentation which should yield critical tests of the efficacy of group protection and of its mechanism should be found in treatment with radiant energy applied as heat, light, or ultra-violet radiation. Treatment with the latter was selected as offering promise of clear-cut results, since Hinrichs (1927) has reported that *Arbacia* sperm are less affected in concentrated than in dilute suspensions (obviously a mass protection), and since Miss Walburga Petersen has obtained similar results in this laboratory in radiating *Paramoecium* (unpublished).

METHODS[1]

Planaria dorotocephala was used as experimental material for a variety of reasons. These planarians are somewhat related to *Procerodes*, which had given the definite group protection from hypotonic sea water described above. They are known to secrete slime and may well give off other organic material into the surrounding water. The slime is known to adsorb particles of colloidal silver and might well serve to protect animals from injury due to other causes. The

[1] I am indebted to the Victor X-Ray Corporation for furnishing the ultra-violet lamp without charge; to Professor C. M. Child, Dr. L. H. Hyman, and Dr. Marie Hinrichs for suggestions concerning the treatment of the planarians.

extended experience of Professor Child and his associates with this species has given us much detailed knowledge concerning their general physiology and laboratory ecology. The experience of these investigators indicates that while water in which these worms have stood for some time has a definitely depressing effect (Child, 1911, 1911*a*), yet distilled water comes to optimum condition more rapidly when there are a number of worms present.

An air-cooled quartz mercury-vapor arc running at 110 volts D.C. and 10 amperes was used as a source. In all experiments the animals were exposed to the full spectrum at a distance of 25 cm. from the source. They were placed in sufficient water to cover the largest, which gave a depth of water of approximately 1 mm. The exposures were made with the dishes placed on a large cement slab exposed to the full blast of a large electric fan. The temperature did not rise more than 4° C. during the exposures, which were usually for 2 minutes but which lasted in different experiments from 1 to 3.33 minutes. The increase in temperature was usually less than 3°, and tests showed that even a sudden increase in temperature of this amount did not affect the survival of the planarians used in these experiments.

All experiments were carried out in a well-ventilated experimental dark room which was usually held at a temperature of 21° C. The extreme temperature range of the room during the entire period covered by these experiments was between 16° C. and 24° C. The first part of the work was directed toward determining the protective action, if any, of "soup" made by cytolyzing the worms under continued exposure to ultra-violet radiation. The following standard procedure was used in this work:

Worms were used which had been starved in most cases for 10 days or more. Enough whole worms or parts of worms were placed in a Petri dish 95 mm. in diameter to make the equivalent of 25 large worms. These were washed through one or more changes of aerated well water and finally exposed in 22 cc. of this water. At the same time a similar Petri dish was exposed containing the same amount of well water but no worms. Both were radiated for an hour under the full spectrum and at the distance of 25 cm. used in all these experiments, with the cooling fan running throughout.

Under these conditions approximately half the water evaporated during exposure, and the temperature rose about 4° C. At the end of this exposure the worms were well cytolyzed. The resulting "soup" was filtered and diluted or boiled, or both, as recorded for the different experiments.

Four equivalent lots of worms were selected from stock and isolated into series of salt-cellar type watch glasses. The worms were then washed and placed in 1 cc. of the clear, aerated well water, which covered the bottom to the depth of about 1 mm. Each worm was examined under the binocular dissecting microscope to insure its being in first-class condition. An equal number of representatives of two of these series was exposed simultaneously to the full ultra-violet spectrum for from 1 to 3.33 minutes, depending on the experiment. Others were exposed similarly until two of the four lots had been equally exposed to ultra-violet radiations.

These radiated worms were examined again under the binocular. The radiated water was pipetted off and replaced in one series by from 1 to 5 cc. of radiated worm "soup" and in the other by a similar amount of radiated water. Typically one of the other lots was placed without radiation in the worm "soup" with each worm isolated in the same amount of "soup" as were the radiated worms. This constituted a "soup" control. Typically the other lot was similarly placed in aerated well water. All the watch glasses were kept in appropriate moist chambers and were inspected from time to time under the binocular microscope.

EXPERIMENTAL RESULTS

1. TRANSFER TO WATER CONTAINING PRODUCTS OF CYTOLYSIS

The results obtained from this treatment are summarized in Table I, which gives a brief record of the results from seven experiments. A similar number of other earlier ones could be listed but are omitted because they were less complete or because methods were being changed. These earlier experiments have affected the conclusions reached only in that they all support the results of those listed here.

An attempt was made to determine the initial appearance and to follow the progress of harmful effects of the treatment. Such observations were recorded by means of descriptive notes made at the

successive intervals of inspection. From these notes, the time before the onset of the first permanent gross morphological changes and the time before the entire worm was affected by the cytolyzing processes have been chosen as representing two definite periods which may be compared fairly in different series in order to obtain information concerning the effectiveness of the different sorts of treatment.

The first morphological effects of radiation frequently appeared as swellings of the lateral margins either just behind the head or at the anterior end of the posterior zooid (Child, 1915). In other cases the first effects of the treatment consisted in the appearance of small lesions on the dorsal surface of the head or at the tip of the tail. When worms had been fed recently, the initial lesions might appear in the region of the pharynx. The first observable effects might be transitory, from which the worm might make a temporary recovery only to succumb to a later similar attack. The mean time before a given lot were first permanently affected is given in the tables under the heading "First Effect."

Once cytolysis began, it showed two main methods of running its course to completion. If the progress of cytolysis was rapid, then the entire dorsal epithelium might become affected and disappear, leaving the ventral epithelium fairly intact and in contact with opaque fuzzy strands of the parenchyma. If cytolysis was slow, disintegration of the entire body at a given level took place at approximately the same time, though the dorsal wall went more rapidly. The four places where initial cytolysis might be expected were, first, the head region or the tip of the tail and, second, the region of the pharynx or the region of the anterior end of the second zooid. From these regions, cytolysis proceeded until the entire worm was disintegrated (cf. Hinrichs, 1924). The mean time required until the entire worm was plainly affected by the cytolyzing processes is given in the following tables under the heading "Cytolyzed." In experiments which extend over so long a period, it is obvious that close watch could not be kept at all times. When significant changes were discovered after an interval of several hours since the preceding observation, they were recorded as having occurred half-way between the two inspections.

In five of the seven cases recorded in Table I, the worms remained intact longer in the radiated water than in the worm "soup." In the two other sets, all the worms of both series were affected when first inspected, so that no differentiation could be made at this

TABLE I

SHOWING THE SURVIVAL OF RADIATED PLANARIANS TRANSFERRED TO WATER CONTAINING PRODUCTS OF CYTOLYSIS*

EXPT.	MINUTES OF EXPOSURE	WATER PLUS CYTOLYSIS PRODUCTS			RADIATED WATER		
		No. of Worms	First Effect (Hours)	Cytolyzed (Hours)	No. of Worms	First Effect (Hours)	Cytolyzed (Hours)
A. Radiated Worms							
11.....	2	10	3.6	55.0	10	39.2	117.1
11a.....	3	5	7.8	44.25	5	10.8	57.6
17.....	2	5	3.4	15.3	5	2.7	12.7
18.....	2	5	1.67	5.75	5	9.8	20.2
19.....	1	6	11.5	29.3	6	17.2	52.5
20.....	2	6	1.45	6.75	6	1.45	6.75
21.....	2	6	0.87	10.85	6	0.87	13.0
B. Non-radiated Worms							
		WATER PLUS CYTOLYSIS PRODUCTS			NON-RADIATED WATER		
11.....	2	5	44.0	72.8	5	62.0	
17.....	2	5	15.0	45.5	5	All intact after 53 hours	
18.....	2	5	5.75	19.0	5	4 O.K. All alive after 5 days	
20.....	2	5	20.2	49.0	5	78	92.5
21.....	2	5	O.K. after 8.0	3 alive after 32		2 O.K. All alive after 80 hours	

* All worms used were 18–20 mm. long.

Notes on conditioned water (worm "soup") used: Experiments 11 and 11a: fifty worms in 10 cc. water. Experiments 17 and 18: twenty-five worms to 20 cc., half of which is added after cytolysis; then all boiled, cooled, and shaken. Experiments 19, 20, 21: the same as 17 and 18 except that only five worms were used.

point. When the mean time for complete cytolysis is taken as a criterion, again in five of seven cases, survival is greater for the worms in the radiated well water. In one experiment, No. 20, there is an apparent tie, in that both lots were found to have cytolyzed in the same mean time. In this case, readings at closer intervals would probably have revealed a greater longevity for the worms in the radiated well water because their cytolysis was less complete at

the time of last inspection than was that of the worms exposed in the worm "soup." In the one other case, worms in the water containing decomposition products showed a greater mean survival period on account of the resistance of one worm. The four others in the set were dead after 6.7 hours at a time when all five worms in radiated well water were still alive.

Different lots of planarians vary so greatly in physiological state that average results from experiments on different days are difficult to interpret. In the present series, in addition to the variation in physiological condition of the worms, the exposure varied for the different lots from 1 to 3 minutes; and there was also a variation in the concentration and the treatment of the worm-conditioned water (worm "soup"). Examination of the difference in time to complete cytolysis in these two series, when examined by "Student's" method (1925) shows that random sampling would have 50 chances in 100 of yielding so great a deviation from the mean in either direction. Obviously such differences have no statistical significance.

Although without statistical significance, it is worth noting that in both comparisons made, the worms placed after radiation in radiated well water fared better than did those placed in such water which contained decomposition products of other worms. Certainly we have here no evidence for a protective conditioning of the water such as was found when *Procerodes* were treated with hypotonic sea water. Such water not only does not aid *P. dorotecephala* in recovery from exposure to ultra-violet but appears to be harmful for worms even if they have not been radiated. This is shown in Section B of Table I. Evidently protection from exposure to ultra-violet radiation requires a different mechanism from that which has been shown to give excellent protection to *Procerodes* when subjected to extreme changes in osmotic pressure.

The worm "soup" had undergone two distinct conditioning processes. In the first place, there had been the exposure for an hour to radiation which might have an effect upon the water regardless of its effect on worms. In the second place, there was the effect on the contained worms which resulted in their cytolysis. In the experiments reported in Table I, the effect of the hour's radiation on the water is checked adequately by running the comparison experi-

ments in water which had also been radiated for an hour but which had contained no worms. There is further evidence that the radiation of this well water for this period under the conditions used did not have injurious effects.[1]

Thus in Experiments 19, 20, and 21 three sets of six worms each were transferred after radiation to non-radiated water, and their survival was then compared with that of worms radiated for a similar time, a part of which had been transferred to worm "soup" and

TABLE II

SHOWING THE RELATIVE EFFECT OF TRANSFERRING WORMS AFTER EQUAL PERIODS OF RADIATION IN 1 CC. WELL WATER TO WATER CONTAINING PRODUCTS OF CYTOLYSIS (WORM "SOUP"), TO RADIATED WATER, AND TO NON-RADIATED WATER, ALL OTHER CONDITIONS BEING HELD CONSTANT.*

EXPT.	WATER PLUS PRODUCTS OF CYTOLYSIS			RADIATED WATER		NON-RADIATED WATER	
	Radiation (Minutes)	First Effect (Hours)	Cytolysis (Hours)	First Effect (Hours)	Cytolysis (Hours)	First Effect (Hours)	Cytolysis (Hours)
19	1	11.5	29.3	17.2	52.5	14.2	31.0
20	2	1.45	6.75	1.45	6.75	1.27	6.0
21	2	0.87	10.85	0.87	13.0	0.83	11.0

* The results are for six individuals in each case. All were from 18 to 20 mm. long.

the others to water that had been radiated for an hour. The results from the worms in the "soup" and the radiated water have already been given in Table I but may be briefly summarized in the present connection in Table II. These tests indicate that the aerated water used was less injurious to the exposed worms after it had been radiated for an hour than was the raw, aerated well water.

2. TRANSFER TO ACCUSTOMED WATER

The water in which planarians have been kept contains secretion and excretion products which, according to the experience of Professor Child (*loc. cit.*), produce certain effects upon planarian head formation comparable to those resulting from exposure to agents known to have a depressing action. When such products are present

[1] Several interested friends have suggested that the radiation may well have altered the pH of the water and that the worms were affected by this change. Tests made during the progress of the experiments showed no change in pH detectable by colorimetric methods in water radiated for 1 hour.

in relatively large amounts, the worms suffer gradual cytolysis. The disintegration and death of the worms in the non-radiated control series in these experiments occurred when the concentration of waste products became sufficientiy high. Water from relatively fresh laboratory cultures represents a medium to which the worms are accustomed. On transfer from it to fresh well water, they show distinct behavior differences. Accordingly it was thought possible that transfer to such water after exposure to radiation might allow a greater survival period and might even favor recovery. Two tests of this possibility were made, with the results summarized below. Here the mean time following radiation before cytolysis affected the entire organism is used as the criterion of the effect of the treatment on survival. There is no evidence of greater recovery caused by transfer to water to which the worms are accustomed. Rather the reverse is true, and the worms are in definitely poorer condition than when placed in radiated well water following radiation. Five worms, 18–20 mm. long, were used in each test. Those in Experiment 12 were radiated for 2 minutes; those in Experiment 13, for 3 minutes. The survival periods were obtained when the worms remained isolated in 1 cc. of water, to which they were transferred immediately following radiation.

In Experiment 12, the cytolyzing processes affected the whole bodies of the worms transferred to culture water in a mean time of 31.5 hours; in radiated well water, the same stage was reached in an average time of 39.1 hours. In Experiment 13, the corresponding averages were 12.2 hours and 15.5 hours, respectively. The mean time taken for the unexposed worms of the control to reach the same stage was 76.4 hours in the culture water and 81 hours in the standard aerated well water.

3. GROUP EXPOSURE

a) *Uncrowded.*—In the different tests recorded thus far, the worms had been exposed singly to radiation while barely covered with clear water. This water was pipetted off immediately following the exposure, and the appropriate liquid was added to the worms which remained isolated throughout. An attempt was made to determine the effect, if any, of radiating groups of animals to see if

while being radiated they might give off some exudate that would affect their subsequent survival.

These experiments were carried out in Petri dishes 93 mm. in diameter. Fifty worms were placed in such a dish. After careful washing, 10 cc. of the usual aerated well water were added. The depth was approximately 1 mm., being about the same as that in the watch glasses in the exposures described above. The worms were spaced as evenly as possible in the dish. During exposure they writhed and twisted in characteristic fashion under the action of the radiant energy. These writhings tended to keep the worms independent of each other, so that there was little overlapping or even contact between different individuals. The effect of the slight drift due to the fan-driven air was overcome by constantly rotating the dish. After exposure, all the animals were left together in the water in which they had been treated.

As soon as possible after radiating such a group, a set of five singles was exposed successively for the same length of time, one worm per dish containing 10 cc. of water. Particular care was exercised here and elsewhere that the position of the containers should be identical in successive radiations. The results from these uncrowded group radiations are summarized below. All the exposures were for 2 minutes. The worms were from 18 to 20 mm. long.

Experiment 14. *Group:* Mean time to first permanent effect, 3.6 hours; mean time until cytolyzing processes have completely affected each worm, 7.7 hours. *Singles:* Similar values, 5.55 hours and 44.3 hours, respectively. The same mean periods for forty-five non-radiated worms placed in 10 cc. of radiated water are 44.3 hours and 52 hours, respectively.

Experiment 15. The same time values for the group of worms are 37.2 hours and 62 hours, respectively. The accompanying radiated singles were first affected after a mean time of 43.2 hours. Only one of the five had undergone cytolysis in 64 hours. The rest were still alive when the experiment closed after 145 hours. Two non-radiated worms placed together in 10 cc. of radiated water were still alive in good condition after 9 days. One showed a small lesion at 112 hours, which later healed. Both had divided, and the resulting parts were undergoing regeneration.

The wide variation in survival in the two experiments is related to the laboratory history of the animals concerned. The worms used in Experiment 14 had stood for some time in a relatively small container; those in Experiment 15 were freshly withdrawn from a large stock culture, following the usual method for these experiments. The results indicate that the latter were in much more vigorous condition. In both these experiments the animals exposed and kept together exerted a harmful rather than a beneficial influence upon each other. Hinrichs (1927) has recorded that exposure of *Arbacia* eggs to ultra-violet radiation increases the rate of certain exudations. If the same holds for planarians—and we may expect radiation to alter the semipermeability of the surface cells (Lillie and Baskervill, 1922)—either the loss of the exuded material by the individual worms or its presence in increased amounts in the surrounding water, or both taken together, are harmful.

It was possible that the decreased survival of the group of radiated worms might come from their standing together in the small amount of water used in the last experiments, where fifty worms were kept in 10 cc. of water until they disintegrated. That the brief survival in Experiment 14 is to be explained entirely as a result of crowding is negatived by the experience of the control group of forty-five in the same amount of water which lived about six times as long as did the exposed group. But was the harmful result produced during the period of group radiation or was it due to remaining in water containing an increasing load of exudates and products of decomposition?

Experiment 30 was run to obtain some indication of the answer to this question. In this experiment, conditions were similar to those in Experiment 15 in every respect except that two different sizes of worms were used. About half of each lot were from 18 to 20 mm. long, and the other half were from 10 to 14 mm. After exposure, as in Experiment 15, pairs of worms—a large and a small one—were transferred to the usual watch glasses containing 5 cc. of aerated, non-radiated well water, and were placed as usual in appropriate moist chambers. Under these conditions, the mean time to the first permanent morphological effects was 12.1 hours. The mean time before the cytolyzing processes completely affected each worm was

40.5 hours. These values are to be contrasted with the mean intervals to similar stages as shown by ten worms selected and exposed similarly but with only two worms present at the time of the exposure or later. The respective mean values are: 12.7 and 44.3 hours. Again there is no indication of protection by group exposure, but the harmful effects of such exposure are less marked. Evidently the early death of the group in Experiments 14 and 15 was due mainly to the accumulation of waste products in the water in which the worms were confined. A control group of ten similar non-radiated worms introduced as pairs of one large and one small worm into 5 cc. of non-radiated, aerated well water showed mean survival times of 44.8 hours for the first effect and 85.6 hours for the final action.

In all the tests reported thus far, group exposure, or the transfer of exposed animals to various sorts of group-conditioned water before or after radiation, has failed completely to show increased survival values. Rather, there have been continued indications of the harmful effect of the group expressed through the shorter survival of both the radiated and the non-radiated animals when transferred to worm-conditioned water.

b) *Crowded.*—Work with the brittle starfish, *Ophioderma brevispina* (Allee, 1927), has indicated that protection might be furnished by massed animals without necessarily involving chemical exudates. These starfishes crawl about among blades of eelgrass in their natural habitat. When placed in smooth-walled glass dishes in the laboratory, physical contact with other individuals or even with glass rods has survival value, other conditions being equal and the mass of animals not too large in proportion to the volume of water. Similar physical protection against loss of water occurs when land isopods (*Oniscidae*) are allowed to form bunches when in a dry container (Allee, 1926). With *P. dorotocephala* exposed to ultraviolet radiation, such crowding should produce physical interference with the penetration of the destructive rays. That this might be protective was indicated by the experience of Hinrichs (1926), who had noted:

> In viewing an exposed lot of sperm with a microscope, it can be seen that the surface layer contains immobile sperm, while the layers below (as seen in

optical section) have been "shaded" from the ultra-violet radiation (or have not been reached because of insufficient penetration of the rays) and are therefore normally active.

In tests made to cover this possibility, from fifteen to sixty worms were exposed in 1 cc. of the standard aerated well water for 2 minutes at the usual distance from the mercury-arc lamp (25 cm.). Particular care was taken that each lot might receive the same radiation as any other lot. For this purpose four watch glasses were exposed simultaneously. One might contain the group, and the other three might contain single worms; or all four might contain single worms only. The glasses were placed around the most intensely lighted point and were shifted at the end of each 30 seconds until each had occupied the four cardinal positions for an equal period during the exposure. After exposure, the worms were inspected as usual. Those radiated together were separated into 3 cc. of the usual aerated well water, and those radiated singly or in pairs were likewise similarly transferred. Here, as elsewhere, when one lot of animals in an experiment was handled, the other lots with which they were to be compared directly were given similar treatment.

The survival after this exposure is summarized in Table III, which is arranged on the same plan as Table I. In all the experiments except No. 28, random selections were made from the exposed group for survival tests. In Experiment 28 the survival of all exposed worms was followed either as No. 28 or 28′. In every comparison made, the mean time taken to reach a given stage was greater when several worms were present during the period of radiation than when only one or two were exposed in the same dish. The fate of the unexposed controls indicates that the effects recorded for the radiated specimens were due primarily to the radiation rather than to some other aspect of the treatment.

Examination of the differences by "Student's" method for determining statistical significance shows that there are 326 chances in 10,000 of obtaining so great a deviation in either direction from the mean as that between the mean time before the cytolyzing processes have completely affected each worm in the two series. Such results have statistical significance.

Since the final survival of the massed animals was approximately

twice that of their isolated fellows, one experiment (No. 29) was tried in which 4 minutes' exposure was given to a group of thirty-three worms in place of the usual 2 minutes. Other conditions remained the same; and in order that the worms might be subject to identical conditions save for the increased exposure, the water was changed at the end of the first 2 minutes of radiation. The tests were

TABLE III

SHOWING THE EFFECT OF GROUP EXPOSURE TO ULTRA-VIOLET RADIATION AS COMPARED WITH THE EXPOSURE OF ONE OR TWO WORMS*

Expt.	No. Worms in Group	Group			Singles		
		First Effect (Hours)	Cytolyzed (Hours)	No. Worms Watched	First Effect (Hours)	Cytolyzed (Hours)	No. Worms Watched
22	15	11.6	50.2	10	2.4	28.7	7
23	60	5.55	40.6	10	1.0	17.3	4
24	60	7.8	29.6	8	1.1	11.8	6
25	30	0.8	8.1	10	0.3	4.0	11
26	60	16.75	70.3	10	10.7	51.3	10
27	60	25.5	61.9	10	6.2	31.1	10
27′	60	16.5	52.1	11	4.8	33.4	11
28	30	16.1	62.5	15	7.4	39.5	13
28′	30	19.7	62.9	15	3.8	30.0	13

Expt.	Unexposed Controls in Aerated Well Water				
	First Effect (Hours)	Range	No. Worms	Cytolyzed (Hours)	Range
22c	21.3	(0.85– 61.0)	5	92.7	(85–108)
24c	65.5	(25.6 –121.6)	5	149.0	(60–190)
25c	114.6	(51.3 –200.0)	5	209.6	(200–242)
28c	134.7	(84.0 –180.0)	5	164.9	(126–193)

* All exposures were for two minutes in 1 cc. of well water in similar watch glasses.

made at the same time as those recorded in Experiment 28 and should be compared with the results of the exposure of those isolated pairs. The initial effects became apparent in a decidedly shorter interval (mean time, 1.1 hours), and the worms underwent cytolysis much more rapidly (mean time, 20.9 hours). Thus, while with equal exposure the final survival is approximately twice as great when numbers are exposed as compared with isolated or paired individuals in the small amount of water and in similar containers, doubling the exposure produces greater rather than equal effects.

4. PRESENCE OF EXUDATES DURING RADIATION

Still another effort was made to test out the effect of exudates upon the resistance to ultra-violet radiation. In Experiments 27, 28, and 29 the water in which the groups had been radiated for 2 minutes at 25 cm. was pipetted off, made up to 1 cc., and transferred to a pair of freshly isolated worms which were then radiated in this exudate for the usual 2 minutes. Here, for the first time in these

TABLE IV

SHOWING THE EFFECT OF RADIATING PLANARIANS WHILE IN WATER IN WHICH GROUPS OF OTHER WORMS HAD JUST BEEN RADIATED

Expt.	Group			Singles or Pairs			Singles in Group Water		
	No. Worms	First Effect (Hours)	Cyto-lyzed (Hours)	No. Worms	First Effect (Hours)	Cyto-lyzed (Hours)	No. Worms	First Effect (Hours)	Cyto-lyzed (Hours)
28.....	30	17.9	62.7	26	5.6	34.8	1	3.25	69.5
							1	0.76	23.5
							1	0.84	38.0
							1	0.84	38.0
							1	2.0	13.3
							1	0.76	6.0
							6	1.31	31.8*
27.....	21	20.8	57.0	21	5.0	32.3	1	0.1	2.33
							1	0.1	2.33
							2	0.1	2.33*

* Mean.

tests, the worms were radiated in water which contained from the start emanations from other worms. The results of these tests are given in Table IV. This water was apparently as clear and transparent as ordinary well water and could be used without the danger of interfering with the penetration of radiant energy which would have been affected had worms been radiated in the worm "soup" even after that preparation had been filtered. For comparison, summaries of the survival of the accompanying lots of worms are also given. Even a casual inspection of the table will show that there was no protection furnished by the presence of the water in which groups of worms had just been radiated.

DISCUSSION

The present report is one of a series dealing with the physiological effects of crowding upon the individuals concerned. The phenomena described in the preceding experimental section demonstrate two facts:

In the first place, the presence of products of cytolysis produced by exposure to ultra-violet radiation are more harmful than beneficial to those worms that have been exposed to the action of ultra-violet radiation or to those that have not been exposed. Similarly, water containing products of metabolism or exudates given off by the animal, either in its usual laboratory culture or when exposed to ultra-violet radiation, shortens rather than lengthens the survival of other worms isolated into it.

In the second place, the exposure of a number of worms in a limited amount of water with limited exposed area, gives much greater protection for the individuals than if they had been exposed singly or in pairs. Some of the implications of these results deserve consideration and will be discussed in the order just given.

When the survival of worms in various sorts of worm-conditioned water is compared with that of similarly treated planarians in aerated well water, the worms in the conditioned water are found to die more quickly. The difference in the survival periods is usually small, and the periods themselves are highly variable for different lots of worms. Eleven such group comparisons are possible with the data at hand; and in these, nine cases definitely favor the well water, one favors the conditioned water, and the other, though recorded as observed, is a tie, with more careful observation it would probably have been placed definitely in the majority column. The main inquiry was concerned with the possibility of there being a protective value in the conditioned water, and there is no doubt of the negative answer given by the experiments. Rather, the converse is indicated.

These experiments have not exhausted the possibility that other sorts of conditioned water might produce beneficial effects. The work of Stedman and Mendel (1926) indicates that the continued radiation of the worms for an hour in preparing the worm "soup" may well have produced toxic substances which might have been absent in worms cytolyzed by some other process, as by exposure to

high temperature. Such possibilities, while recognized, did not enter into the present problem, which is concerned with the inquiry as to whether or not exposure of a group of planarians to some physical agent (ultra-violet radiation in this case) would favor the survival of any or all of the group, as contrasted with the similar exposure of isolated or paired worms.

Neither do these experiments demonstrate the nature of the physiological processes which hasten the disintegration of the radiated worms. Several possibilities exist; for example, the observed cytolysis may be due to the direct action of the radiant energy upon the tissues. On the other hand, it may act primarily by altering surface permeability (Lillie and Baskervill, 1926), thereby increasing sensitiveness to changes in pH or other chemicals in the environment or to the lowered concentration of substances within the body (Hinrichs, 1916, 1917). Any or all of these processes may affect the organism by upsetting the neutrality or osmotic equilibrium. These are important questions, but they lie beyond the scope of the present inquiry.

In running the experiments, no particular attempt was made to secure uniformity from day to day in the physiological condition of the planarian worms, the only care being that the different lots making up one experiment might be similar. Under these conditions the statistical significance of the observed difference in length of survival of the worms in the conditions under discussion is not high but is in general in agreement with the results of previous workers with similarly conditioned water. Thus Child (1911, p. 203) shows that old culture water definitely depresses normal head formation. He states (1911*a*, p. 132):

> Similarly the presence of metabolic products of Planaria in the water undoubtedly decreases the rate of metabolism and the effect on regulatory morphogenesis is similar to that of starvation or low temperature, though it may be greater and in extreme cases it approaches that obtained with the anesthetics.

More recently Goetsch (1924), with similarly good experimental technique, found that the rate of growth in *Planaria lugubris* is retarded by the presence of its own excretory products. Excreta of related animals in the concentrations used also retards growth-rate, but those of unrelated animals have no effect.

The results obtained in my own experiments are not only in accord with these earlier conclusions but may serve to connect them with the observations of Drzewina and Bohn (1921, 1922), who observed that the planarians *Convoluta* and *Polycelis* in the presence of KCl give off a substance which leads to their own destruction and which they regard as being definitely autodestructive. This destructive substance is reported to be present in worm-conditioned water. They contrast this situation with the greater protection furnished by conditioned water to animals exposed to suspensions of colloidal silver, which they regard as due to the presence of an autoprotective substance.

One cannot be sure of the nature of the substances that are acting in my experiments in the case of water containing cytolyzed worms. The observed effects may be due to bacterial decomposition of the products of cytolysis; or, as noted above, these products may have been broken down by the direct action of the ultra-violet radiation into toxic materials. At any rate, these substances do not need to be specific autodestructive agents and probably are not such. Rather the process of autodestruction described by Drzewina and Bohn, and observed to some extent at least in the present experiments, appears to be a more rapidly acting and therefore more dramatic expression of the old, well-known fact that masses of animals frequently produce conditions that are harmful to the crowded animals—a fact discovered by Hogg (1854), worked with by Semper (1874), identified as a property of old culture water by Child (1911), and shown to be due to the presence of excretion products by Goetsch (1924).

Further, it is interesting to note that conditioned water such as was found to be definitely protective when *P. dorotocephala* was exposed to suspensions of colloidal silver is either non-protective or harmful when similar animals are exposed to ultra-violet radiation.

In the former case, the presence of organic material in the water served to remove (probably by adsorption) the colloidal silver that was yet more toxic; but with the colloidal silver absent, the possible toxicity of the organic matter itself has time to develop. Despite the indications from these experiments, the evidence is not complete concerning the possibility that with different water and with smaller

proportions of the conditioning matter, such conditioned water may prove advantageous to the worms placed in it.

With regard to the definite protection furnished when many worms were exposed to ultra-violet radiation in a limited amount of water and with a limited surface area, the experimental evidence indicates that this protection is due to some sort of interference with the penetration of injurious rays rather than to the presence of some exudate or exudates released as a result of the radiation. As has been stated above, water containing such exudates produced harmful rather than beneficial results.

In the experiments where the worms were more densely crowded (sixty to the cubic centimeter of water), the protection was obviously connected with the "shading" which Dr. Hinrichs mentions in her studies on the radiation of sperm suspensions. In the less dense groups (fifteen to the cubic centimeter), "shading" in the usual sense is less obvious; but exposure at this density also resulted in definite protection to the group-exposed worms, which suggests that some other factor or factors may have been operating. Similar shading would undoubtedly have resulted from the exposure of isolated worms in water conditioned by the pressure of products of cytolysis. The experiment was not tried, since the only information to be gained would have been the relative value of the protection and the toxicity of such conditioned water.

It is worth noting that all the members of the group exposed together were benefited by the fact that they were together. In such circumstances it might well have happened that certain animals at the surface would take the brunt of the harmful rays. Their presence might serve to protect the other members, and the group might thus be of value to the species if not to all the individuals composing it. In Experiment 28, where the subsequent history was taken for all the exposed animals, the interval before the first effects of exposure was observed in the thirty animals exposed as a group ranged from 27 minutes to 32.8 hours. Their final cytolysis ranged from 8.25 to 106 hours. The same conditions were observed for the twenty-six worms exposed in pairs in from 1 minute to 23.5 hours, and from 3.4 to 84 hours, respectively. Thirteen (50 per cent) of those exposed as pairs were visibly affected before the end of the first hour after

exposure, while six (20 per cent) of the grouped animals were similarly affected in the same time. Six of those radiated in pairs were dead before the first of the group died.

I am not prepared to generalize widely from these experiments with the relatively large and highly pigmented *P. dorotocephala* that mass protection from ultra-violet radiation is always due solely to some sort of physical interference rather than to the possibly protective action of some exudate. With small organisms such as sperm of sea urchins or with *Paramoecia*, which are more translucent, the mass protection may be due to the latter factor, as Hinrichs suggests. Her observations show, however, that even with such minute organisms, the fact of physical interference is significant.

Single planarians, and to a greater extent massed planarians, would be more nearly analogous to sperm or protozoan aggregates than to a suspension in which distribution is fairly equal in three dimensions, and each organism is surrounded by a medium of approximately uniform consistency. In the latter case, it is conceivable that a closer equilibrium between cells and medium must be maintained than with larger forms. This subject is one for experimentation rather than a priori discussion.

In general the experiments recorded in this report forward our understanding of mass relations of individuals in two ways: First, the results emphasize the fact that the phenomena of possible protection of individuals by chemical exudates, which has been demonstrated for many animals exposed to diverse situations, is not universal. Second, they show that even when the massed animals are known to produce chemical exudates which are harmful, the massing may still have survival value through the changed physical conditions which it produces.

SUMMARY

1. The flatworm, *Planaria dorotocephala*, is not protected from the harmful effects of radiation with the full spectrum of a quartz mercury-vapor arc by transfer after exposure to water containing worms cytolyzed by radiation, or to culture water containing excretory products and disintegrated slime.

2. Planarians are not protected from the effects of such radiation by being radiated in water containing exudates given off by groups of worms similarly exposed.

3. In both cases the effects of such treatment are harmful rather than beneficial.

4. There is definite protection from the action of such radiation furnished by exposing a large number of worms in a small volume of water having restricted exposed surface. Such protective effects appear to be due to physical interference comparable in many respects to shading from ordinary light.

5. Masses of individuals may have survival value for all the individuals comprising the mass despite the fact that the concentration of animals produces a somewhat toxic concentration of exudates.

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CHANGES IN THE OSMOTIC PRESSURE AND WATER CONTENT OF CRABS DURING THE MOLT CYCLE

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IT HAS long been known that crabs increase greatly in size when molting takes place. This increase in size occurs in a very short time (about half an hour) and is due to the taking up of water. Olmsted and Baumberger (1923) have discussed this increase in size, showing that amounts to between 30 and 40 per cent of the previous volume in certain species and that corresponding changes in specific gravity and water content occur. Since the mechanism of the intake of water during molting has not been satisfactorily explained, as far as we know, the following investigation was undertaken to study this phenomenon.

It appeared to us that a study of the osmotic pressure and hydrogen-ion concentration of the blood would be most likely to give a clue to the question of how the organism is able to take up so large a quantity of water in a very short time.

The species studied were the Grapsoid crabs, *Pachygrapsus crassipes*, *Hemigrapsus oregonensis*, and *H. nudus*. In all our experiments where no species is mentioned we have used *Pachygrapsus crassipes*.

METHOD

Osmotic pressure was determined by the freezing-point depression method, described by Jones (1897), using a Beckmann thermometer. The fluids of the crab were obtained by placing the animal in a thick glass tumbler and squeezing it under a heavy glass cylinder. The volume of liquid which could be drained off was

[1] The experiments described in this paper were performed during the summer of 1920 and 1921 at the Hopkins Marine Station of Stanford University, located at Pacific Grove, on Monterey Bay, California. We wish to thank Dr. W. K. Fisher, director of the laboratory, for his efforts to facilitate our work, and to our colleagues Drs. E. G. Martin and F. W. Weymouth for helpful suggestions.

measured and brought up to 10 cc. with distilled water. We found that this dilution did not appreciably increase the dissociation of the substances in the blood, for the osmotic pressure remained the same when corrected for the dilution. The hydrogen-ion concentration was determined by colorimetric means, as described by Clark (1920).

THE MOLT CYCLE

Six stages may be recognized in the crab during the molt cycle. These conditions will be called (1) "hard," (2) "pillans," (3) "about to molt," (4) "newly molted," (5) "soft," and (6) "paper shell." *Hard crabs*, as the name implies, have a firm exoskeleton; they are extremely active and are in the middle of the molt cycle. The duration of the hard condition is much longer than any of the other stages, and its duration varies with the size of the crab, being longer with large crabs, due to the decrease in frequency of molting which accompanies increase in age. The *pillans crabs* have a grayish appearance, their carapace is friable and readily cracks and may be picked off in small pieces, so that they are sometimes called "peelers." The crabs in this stage are rather inactive and live largely under stones in tide pools. The duration of this stage is probably only a few days (3 or 4), during which the crab gradually approaches a more and more fragile condition and finally is ready to molt. The *crab about to molt* may be seen to have partially withdrawn its appendages from the exoskeleton of the legs, and a transverse split in the old exoskeleton has taken place at the junction of the carapace and the tergum of the first abdominal somite, extending forward along the pleural groove and showing the new chitinous carapace beneath. The crab was usually in the act of "backing out" through this slit when the determinations described below were made. The duration of this stage is usually less than an hour. The *newly molted crabs* were individuals which had completely emerged from the cast not more than 15 minutes before a determination was made. These crabs are so soft that they cannot run actively but require the buoyancy of sea water to support them. They quickly lose this fragility and probably do not remain in this condition more than an hour or two. *Soft crabs* already have the tips of the ambulatory appendages hardened and are sufficiently rigid to run freely out of

the sea water. This condition may persist for 12–24 hours before the *paper shell* stage appears, when the crabs have a papery texture and already have the chelae hardened sufficiently to pinch slightly. The cycle is completed when the paper shell has become hard by the gradual deposition of lime in the chitinous exoskeleton. Hay (1905) gives a very accurate description of molting in the blue crab; Pearson (1908), in *Cancer;* and Herrick (1895), in the lobster. The last two authors give many references to observations on this process, and in general these descriptions fit in very closely with our own observations.

The reliability of the grouping of the data given for the different stages depends on the correct identification of the various stages. From the description above, it can be seen that one stage blends into the next to some degree and that there are probably progressive changes in any one stage. It may be said, however, that a specimen which had just been collected could be placed in its group quite accurately, as we learned to recognize these stages by following in the laboratory the molt cycle of many individual crabs which had been classed as "pillans," etc., and noting the character of each succeeding change. A further check on our classification consisted in measuring the specific gravity of the individual in question, for it was found that this was a very constant factor for each stage. We feel that there can be no reasonable doubt of the grouping of the hard, pillans, paper, and about to molt; and naturally the history of newly molted crabs puts their classification beyond doubt.

HYDROGEN-ION CONCENTRATION

Blood was withdrawn from living crabs in the various stages and the pH determined by adding phenol red and comparing with standard buffer tubes or the color chart of Clark (1920). The blood was withdrawn by inserting a pipette into the pericardial sinus at the junction of the carapace and first abdominal sclerite. Blood flowed readily into the pipette and was blown into a tube and the indicator added. In many cases the blood was diluted by adding neutral distilled water, and it was found that this dilution did not appreciably change the pH. Our method was probably accurate to 0.1 pH.

The pH was determined for the blood of a number of hard,

pillans, and soft crabs; but the average of the determinations was very nearly alike for the three stages. However, the variation in pH of the soft crab was great, and this may indicate a characteristic change in pH at some stage in the molt cycle which we failed to observe. The average pH of the blood of crabs is about 8.2; sea water is slightly more alkaline, with a pH of 8.4. The pH determinations were as follows: ten hard crabs 8.1–8.4, average 8.24; seven pillans 8.1–8.4, average 8.18; twelve soft crabs 7.8–8.5, average 8.25. From these data we are unable to show any very definite connection between the pH of the blood and the molting process, although the low pH of the blood of some soft crabs lends support to the hypothesis, given later, that lactic acid may be produced to a marked degree during the act of molting, as does likewise the low CO_2 content described below.

The carbon-dioxide content of the blood was determined by the Van-Slyke and Cullen (1917) alkali reserve method, the blood being drawn from the crabs as described above and introduced directly into the blood-gas pipette so that the tension of CO_2 was probably the same as that existing in the body of the animal. The volume of CO_2 given off by 1 cc. of blood varied considerably in each of the stages, so that great importance cannot be attached to the difference between the averages of the different groups. We have therefore been unable to demonstrate any definite characteristic difference between the alkali reserve of different stages in the molt cycle, although there was indication that the blood of the soft crab might have a lessened CO_2 capacity. The average number of cubic centimeters of CO_2 at 0°, 760 mm. per 100 cc. blood, for hard crabs was 40.4; for pillans, 40.4; for soft, 31.9; and for paper, 38.5. Collip (1920) found 24.2 cc. CO_2 in the blood of *Hemigrapsus nudus* when equilibrated with air.

SPECIFIC GRAVITY AND WATER CONTENT

The specific gravity of crabs changes greatly during the molt cycle. The hard crab, partly because of its calcified exoskeleton, has the highest specific gravity (1.193); and the newly molted crab the lowest (1.038). The data are given in a previous paper (Olmsted and Baumberger, 1923). The specific gravity of the pillans (1.129)

is a little lower than that of the hard crab, possibly because of some loss of material from the carapace, which is very fragile, but more probably because of replacement of tissue by water. That this is apparently the case is shown by the fact that the water content increases; and since the animal is inclosed in a practically non-distensible exoskeleton so that its volume cannot increase greatly, the added water must replace tissue metabolized. The animal is probably living on its own tissues at this time, as it is unable to obtain food as readily as normally because of its fragile condition. This is supported by the fact that on picking off the carapace, the body of the crab appears very much shrunken, the new chitinous carapace being puckered, not smooth and distended. This shrinkage is an advantage to the crab in molting, as it would better enable the animal to withdraw its appendages from its exoskeleton. Weymouth (1917) has compared the "meat weight" of "light" (pillans) and hard crabs of the same size and found in the former that this was 39 per cent of the total weight and in the latter 45 per cent.

The very low specific gravity of the newly molted crab is due to two factors: the casting of the exoskeleton, which makes up about 11 per cent of the weight of a hard crab; and the absorption of a large quantity of water. Crabs in passing from the soft condition to paper shells have increased in specific gravity (to 1.057), probably because of the absorption of calcium in the hardening of the exoskeleton, and by replacing water with newly formed tissue of a density greater than unity. Hecht (1914) has shown that the calcium deposited in the new exoskeleton is not present in the body of some species of newly molted crabs but must be absorbed from food or sea water, whereas Paul and Sharpe (1916) showed that in *Cancer* there was a storing of calcium in the liver. In the species we used, if the carapace of pillans crabs and hard crabs of the same size are treated with HCl, the flexible carapace remaining weighs much less in the case of crabs in the pillans condition. The hard-crab carapace by acid treatment loses about 79 per cent of its weight, while the carapaces of pillans or of cast exoskeletons lose about 93 per cent of their weight. Apparently some substance insoluble in acid, probably chitin, has been removed from the carapace, and presumably from the whole exoskeleton, during the pillans condition or at the

time of molting. Pearson (1908) points out that resorption of calcium along definite lines enables the molting crab to withdraw the chelae from the exoskeleton. The resorption of material from the carapace may be the cause of its extreme fragility during the pillans condition.

The change in water content is one of the most characteristic changes in the crabs during the molt cycle and is an important factor in bringing about the changes in specific gravity that occur. The dry weights of the different stages of three species of crabs are given in our previous paper (Olmsted and Baumberger, 1923). The water content is lowest in the hard crabs and increases only slightly in the pillans; it increases greatly in the newly molted crabs and then gradually decreases until the hard condition is reached. Roughly the figures are: hard, 67 per cent; pillans, 76 per cent; newly molted and soft, 87 per cent; paper shell, 82 per cent. As judged by the ease with which blood can be drawn, the blood volume of the newly molted, soft, and paper shell crabs is very much greater than the blood volume of the hard or pillans. Apparently the water added at the time of molting is largely added to the body fluids. In the pillans, the extra 9 per cent of water must go largely into the tissues, as the blood volume does not seem to be increased. Paul and Sharpe (1916) give the following data on blood volume of two crabs of the same size: 27 cc. in a crab several weeks after molt, and 9 cc. in a crab of the same size approaching molt; a crab 10 days after molt had ten times as much blood as a crab of equal size 1 day before molt. To quote Vitzou (1882, p. 551):

> Dès l'instant où commence la désarticulation et jusqu'au rejet de l'ancien tégument, il s'écoule deux ou trois jours; dans cet intervalle l'animal est énormément gonflé par la quantité d'eau qui traverse par endosmose les nouvelles enveloppes et s'y mêle aux liquides de l'organisme: la quantité de la lymphe est beaucoup plus considerable et moins coagulable, étant plus étendue d'eau à l'époque de la mue qu'à toute autre époque.

Some experiments we performed on blood coagulation show that the body fluids are greatly diluted by the water absorbed at the molt. Using a Dale coagulometer, the blood of hard crabs coagulated in an average of 17 seconds. This coagulation is the result of the substances escaping from the explosive blood corpuscles (Tait, 1910).

In the blood of a late paper-shell crab coagulation took place in 26 seconds on an average. The blood of soft crabs did not coagulate, probably because the comparatively few cells in the given volume of blood all adhered to the shot used in the test, and could not interlace to form a coagulum. Observations under the microscope showed that the cells in the soft-crabs' blood exploded but were very much fewer in number than in the blood of paper-shell or hard crabs.

INCREASE IN SIZE IN MOLTING

We have gone very fully into the data available on this subject in our first paper (Olmsted and Baumberger, 1923), but for completeness it should be said that at the molt crabs increase in width on an average of 9.4 per cent; in volume (including cast), on an average of 42.2 per cent; and in weight (including cast), on an average of 33.9 per cent. This increase in size, weight, and volume takes place in a few hours. That increase in size on molting is the result of absorption of water by the animal seems to us to be proved by the changes in water content and specific gravity which we have described above and by the speed with which the change takes place. It remains, however, to explain by what means the animal can absorb this water and how the absorption is so nicely timed to the needs of the organism.

OSMOTIC PRESSURE

The quick changes in water content described above would naturally be expected to be correlated with changes in osmotic pressure of the tissue fluids of the crabs during the molt cycle. We determined the freezing-point depression in a number of crabs in different stages and soon found that there was a consistant difference. The short duration of the condition—about to molt—prevented us from noting during the first season (1920) the great increase in osmotic pressure that takes place very suddenly in this stage; but in 1921 we made it a point to hunt for crabs in this condition and to hurry with them to the laboratory in order to determine immediately the freezing-point depression. It was only by this quick action that we finally obtained sufficient data on crabs about to molt to prove that the dilution of the body fluid which takes place on molt-

ing is proportional to the change in osmotic pressure which occurs at the same time. The data on freezing-point depression are given in Table I. Several determinations of freezing-point depression were made on the juice from each crab, and these determinations checked within 0.02°. The frequencies given in column 2 of Table I are therefore the number of observations, each of which is based on the average of several determinations on a specimen of fluid from one crab.

TABLE I

OSMOTIC PRESSURE CHANGES

Condition	Frequency (No. Specimen)	Mean Freezing-Point Depression	Standard Deviation	Probable Error of the Mean	Equivalent Molecular Concentration of an "Ideal" Compound	Difference of Means	Probable Error of the Difference	Difference / P. E. D.
Hard	11	1.327	0.10	0.02	0.71	.566	.222	2.5
Pillans	17	1.893	0.37	0.22	1.02	.708	.26	2.7
About to molt	16	2.601	0.82	0.13	1.4	.408	.144	2.8
Newly molted	11	2.193	0.25	0.05	1.18	.049	.07	0.7
Soft	11	2.144	0.25	0.05	1.15	.384	.245	1.6
Paper	10	1.76	0.2	0.04	0.94	.....	.....	.....
Sea water	.....	1.975	.....	.....	1.06	.....	.....	.....

The frequencies are really too small to apply statistical methods with dependability; however, the results of such analyses are suggestive.

In Table I the differences between the means of freezing-point depression and the probable error of the difference are given. When the P. E. D. is contained 2.5 times or more within the difference, the chances are very great that the difference between the means is real and not merely the effect of random sampling from two inadequate populations. According to this criterion, our data are sufficient to prove that there is a difference in osmotic pressure between the different stages in the molt cycle, with the exception of the newly molted, soft, and paper-shell stages, which are not so sharply de-

fined. Thus $\frac{D.}{P.E.D.}$ is 2.5 or greater when paper shell and hard, pillans and hard, about to molt and pillans, and about to molt and newly molted are compared. Furthermore, the differences in freezing-point depression are all in the direction that would be expected if we consider the differences in water content at different stages of the molt cycle and assume that water absorption is due to osmotic pressure. A series of differences, all conforming to a logical scheme, lend strong support to the reality of each.

If we convert freezing-point depression to its equivalent molar concentration of an ideal compound on the basis of $1.86° = 1$ mol., we obtain the figures in column 6 of Table I. This table shows that the osmotic pressure of hard crabs is less than that of sea water, being 0.71 mol., while sea water at Pacific Grove, on Monterey Bay, is 1.06. At Woods Hole, Massachusetts, the freezing-point depression of sea water is 1.9° (Mathews, 1916), or 1.03 mol. Bottazzi (1908) gives the freezing-point depression of the blood of a spider crab as 2.36°, of a lobster as 2.29°, and of the sea water in which these animals lived as 2.3°. These are each about 1.2 mol. He believes that Crustacea are unable to regulate their osmotic pressure but vary with the osmotic pressure of the environment. Our observations are not in accord with this hypothesis. Bottazzi does not state what condition the crabs were in at the time the determinations were made, but our observations show that the crab's osmotic pressure changes in spite of the fixed osmotic pressure of its environment. Thompson (1917) and Martin and Wilbur (1921) have shown that the salt content of the brine shrimp (*Artemia*), which lives in brine ten times as concentrated as sea water, is less than the brine in which they live. Therefore the brine shrimp and the crab have some power of regulating the osmotic pressure of the body fluid regardless of that of the environment. It was found that the pillans crabs had a freezing-point depression of 1.893°, which is only 0.08° less than sea water and 0.5° greater than the hard crabs. The crabs about to molt are 0.6 greater still, and thus much above sea water. The newly molted crabs dropped 0.5°, and after the molt the soft crabs gradually diminished in osmotic pressure until in the paper-shell stage a depression less than sea water is reached.

The decreases in osmotic pressure during the molt cycle can most readily be explained on the basis of water absorption. Thus the osmotic pressure (and from now on we will speak of it in terms of molar concentrations) in the crab about to molt is 1.40, and the water content is (assuming it is the same as in the pillans) about 73 per cent. The newly molted crab has an osmotic pressure of 1.18, and the water content is 85 per cent. Starting with an osmotic pressure of 1.40 and increasing the water content from 73 per cent to 85 per cent, the osmotic pressure (X) may be calculated for the newly molted crab as follows:

$$X=\frac{1.4\times 73}{85}=1.20\,.$$

This calculated value is very close to the osmotic pressure actually observed (1.18), and thus our water-content determinations serve as a check on the freezing-point observations. In the calculation above, we have assumed that pure water was absorbed. If the increase in water content represented salt water absorbed, the salts of which were retained, the osmotic pressure (X') of the newly molted crab would be much higher than is actually found to be the case. This is shown in the following:

$$X'=\frac{1.4\times 73}{85}+\frac{1.06\times 12}{85}=1.35\,.$$

This calculation would indicate that pure water is absorbed on molting or that the salts are excreted. The water absorbed during molting is largely retained in the body when the crab becomes hard, as can be shown by the following calculation:

Condition	Specific Gravity		Water Content	Water per Unit Volume
Hard	1.193	×	0.688	=0.821
Soft	1.038	×	0.845	=0.877

A soft crab has about 88 per cent of its volume occupied by water, while a hard crab has about 82 per cent; and the volume of blood in the soft crab appears to be several times as great as in the hard crab; therefore the water of the blood must enter the tissues as the crab hardens. The tissues do not become more watery, but rather the cells that have rapidly proliferated previous to the molt (Paul and

Sharpe, 1919) gradually increase in size so that the space occupied by blood must be diminished.

The experiments of Lim (1917–18) and Tait (1916–17) appear to support our findings in regard to changes in osmotic pressure. Lim studied the survival of crabs placed in aerated distilled water, and found the period of survival was longer with hard than with pillans crabs. Tait performed similar experiments on a Crustacean (*Ligia*). This animal molts the exoskeleton of first one end of the body and then of the other. Tait found that the animals survived much longer (about 22 hours) after completely molting than they did if approaching a molt (13 hours survival). It seems to us that the results of Lim and of Tait can be explained on the basis of a greater osmotic pressure immediately previous to molting resulting in the more rapid absorption of water and the earlier onset of a fatal oedema.

Changes in osmotic pressure of tissue fluids may commonly occur in growth, judging by the changes in water content referred to by Thompson (1917); and it may be that this phenomenon is merely exaggerated in Crustacea on account of the fact that "growth" is limited to a short period in these animals.

DISCUSSION

We have shown that the osmotic pressure in crabs varies during the molt cycle in such a way as to account for the absorption of water by crabs at the time of molting. Judging by the reduction in osmotic pressure that takes place when water is absorbed, this water must be free from salts or the salts entering with the water are excreted. The question arises as to the place of entrance of the water. Cuenot (1893) had shown that the volume and chemical composition of the blood was regulated by the stomach and liver and that during ecdysis a large quantity of water was absorbed probably through the digestive tract. Paul and Sharpe (1916) showed that the calcium content of the blood remained at the same level in spite of a great change in blood volume after the crab had molted. The constant calcium level in the blood would be an advantage to the organism in maintaining the normal semipermeability of the membranes. The amount of water absorbed would vary with

the osmotic pressure of the blood. Changes in the level of salt excretion by the kidney during the molt cycle might be a factor in raising the osmotic pressure preparatory to molting, but we have no evidence to bear out this possibility. The change in osmotic pressure takes place very quickly, as we found by direct measurement; and Paul (1914) has shown that pillans crabs will not absorb water if the shell is picked off, and we are able to confirm this statement by observation on several late pillans. It appears that some means of quickly raising the osmotic pressure must be available. One of us intends to follow up this problem and try to determine what substance is responsible. It is suggestive that immediately before molting the glycogen content of the crab hepato-pancreas is very high, according to Claude Bernard (1877, 1879) and Vitzou (1882, p. 568). Schönborn (1910, p. 536) found 1.0, 0.85, and 2.2 per cent in *Carcinus maenas* before molting and 0.4 per cent (p. 539) immediately after the molt. Hoet and Kerridge (1926, p. 117) found 0.55 per cent in hard *Cancer pagurus* and less than 0.03 per cent in soft specimens. This glycogen, if suddenly converted into glucose, would very greatly raise the osmotic pressure; and finally, during the struggle of the animal in ecdysis, the glucose could be broken up to lactic acid and increase the osmotic pressure still further. As the sugar was removed, more glycogen would be converted into glucose, the struggle of ecdysis resulting in an increase in blood sugar, as in the convulsions of an insulinized rabbit. This picture gains some support from the observations of Hemmingsen (1924) that the crayfish shows a blood-sugar curve similar to man in experimental hyperglycemia and that the gills are not permeable to glucose even when the blood sugar rises to 0.27 per cent. Also Paul and Sharpe (1919) found a great storage of fat in the liver preparatory to the molt, and a conversion into glycerol and fatty acid after the molt. This could also be a possible mechanism of increasing osmotic pressure.

CONCLUSIONS

1. Crabs increase in volume 30–40 per cent on molting, by the absorption of water. This increase in volume takes place in a very short period of time.

2. The stages in the molt cycle were found to be six in number, recognizable by differences in the integument. These stages are

called "hard," "pillans," "about to molt," "newly molted," "soft," and "paper shell."

3. There is no definite change in the pH or alkali reserve of the blood during the molt cycle.

4. The water content and specific gravity are different in the various stages and are typical for each. The hard crabs have a low water content, pillans higher, and newly molted much higher; the water content then drops off in the later stages. The high water content of the soft crabs develops very quickly at the time of molting, by a large absorption of water. The specific gravity varies inversely as the water content.

5. The osmotic pressure of sea water from Monterey Bay is 1.06; of tissue fluids of hard crabs, 0.71; of pillans, 1.02; of crabs about to molt, 1.40; and of newly molted crabs, 1.18. The absorption of water that takes place accounts for the change in osmotic pressure from 1.4 mol. in the crab about to molt to 1.18 mol. in the newly molted crab.

6. The probable mechanism for changing the osmotic pressure is discussed.

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A STUDY OF THE GLYCOGEN AND SUGAR CONTENT AND THE OSMOTIC PRESSURE OF CRABS DURING THE MOLT CYCLE

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THE sudden increase in size of Crustacea when the hard exoskeleton is shed has been the subject of much study. A review of most of the literature is given in the papers of Olmsted and Baumberger (1923) and of Baumberger and Olmsted (1928). In Naples one of us measured three *Carcinus maenas* within an hour after the molt and found the percentage increase over the width of the cast to be 13, 18, and 23 in crabs of about 3 cm. Thirty specimens of *Pachygrapsus crassipes* measured by Olmsted and Baumberger (1923) averaged 8.5 per cent increase in width, 40.7 per cent increase in volume, and 33.9 per cent increase in weight, the increment having a negative correlation with initial width. Hay (1905) measured nine newly molted female blue crabs, *Callinectes sapidus*, and his figures show an average increase of 23 per cent over the width of the cast.

Osmotic pressure differences were found in the different stages of the molt cycle by Baumberger and Olmsted (1928) in *P. crassipes*, and it was thought advisable to check these results by determinations of freezing-point depression on the expressed fluid of other species of crabs. Crisfield, Maryland, as the center of the soft-shell-crab industry, was selected for the purpose. In the summer of 1925 one of us determined by cryoscopic means the osmotic pressure of several blue crabs, *C. sapidum*. The stages of the molt cycle were recognized by "signs" which have been described by Hay (1905, p. 411) and which are in general similar to the changes in appearance noted by others. The following four determinations of freezing-point

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depression (Δ) were made: (1) a female in the earliest stages of actual withdrawal from the cast, 1.885°; (2) a male about one-eighth withdrawn from cast, 1.665°; (3) a soft male fully emerged 1 hour before determination, 1.225°; and (4) a hard male showing no signs of approaching the molt, 1.380°. In general these determinations agree with those made on seventy-six *P. crassipes*, in so far as Δ is much greater in the crabs in the act of molting (2.601°) than before (1.327°) or after (2.144°) the molt. The fact that Δ for *C. sapidum* are much smaller than for *P. crassipes* possibly bears some relationship to the fact that Δ for the water in which were anchored the fishermen's barges containing the blue crabs was only 1.110°, whereas Δ for the sea water (of Monterey Bay) from which the *P. crassipes* were taken was 1.975°.

Baumberger and Olmsted (1928) came to the conclusion that the high osmotic pressure of the molting crab accounts for the absorption of water that takes place at that time and which results in the increase in volume. These authors suggest that the conversion of stored glycogen to sugar and lactic acid at the time of molt may be an important factor in the increase of osmotic pressure that takes place. A study of the glycogen and sugar content of the blue crab, *C. sapidum*, was therefore made. Specimens in all stages were collected in June, 1925, at Crisfield, Maryland. The animal was submerged in five times its weight of 95 per cent ethyl alcohol, ice cold; the digestive tract was quickly removed and discarded; the hepato-pancreas was transferred to one vessel of ice-cold alcohol; and the balance of the crab was quickly passed through a meat-grinder and collected in a vessel of ice-cold alcohol. Three individuals of the same sex and condition were collected. The stages recognized were the hard crabs, crabs approaching to within a few days of molting, crabs that would molt in a few hours, crabs in the act of molting, and crabs that had molted not more than 1 hour before. The analyses of glycogen and sugar were made in December, 1925. As the specific gravity and water content and total solids of the crab change greatly during the molt, it was finally decided to refer the glycogen and sugar determinations both to the total nitrogen and to the solid bases rather than to the original weight. And considering the fact that the exoskeleton makes up a

large proportion of the total solids, it is clear that the total nitrogen is the most dependable basis of comparison.

The samples of the same stage and sex were composited and then ground in a large mortar. By thoroughly shaking the container before drawing a sample, it was possible to get a fairly uniform suspension of the finely divided tissue. Samples of from 10 to 20 grams of this mixture were used for determination of nitrogen and of solids. A 50-gram sample for sugar and glycogen determination was transferred to a filter, and after filtration was washed three times with 60 per cent alcohol. The insoluble residue was transferred to a beaker, digested with 50 per cent potassium-hydroxide solution, and glycogen was determined by the Pflüger method. The filtrate and washings were evaporated to small volume to remove alcohol, diluted with water to about 30 cc., and protein was precipitated by the Folin-Wu method for blood. After making this up to volume and shaking thoroughly, it was filtered, and an aliquot used for sugar determination by the Folin-Wu method.

It was not possible to determine accurately lactic acid in these samples because of the presence of interfering substances (aldehydes, etc.) in the alcohol used as a preservative.

The anomaly in the results consists in the low sugar and comparatively high glycogen figures in male crabs approaching molt. This characterizes both the muscle and the hepato-pancreas. It is improbable that this anomaly can be due to any kind of experimental error. It is hardly possible that all the other samples were handled with less dispatch and that nearly all their glycogen was converted to sugar before the enzyme was precipitated by alcohol.

The sugar/nitrogen ratios in the hepato-pancreas are fairly concordant and show an undoubtedly increasing trend throughout the series. The same ratios for the muscle are less concordant and show no distinct trend in either direction.

No definite carbohydrate reserve is indicated except in the case of males approaching molt. The increasing sugar content of the hepato-pancreas through the molt cycle would indicate a mobilization of sugar possibly from fat or protein in the absence of glycogen, which possibly was depleted during the sojourn of the crabs on the fishermen's barges. Schönborn (1910) has shown that glycogen is

absent from *Carcinus maenas* starved 10 days, although present up to 2.2 per cent of the wet weights when richly nourished.

In 1924 one of us, using the same methods, determined the glycogen and sugar content of *P. crassipes* immediately after killing. The results in percentage of wet weights are given in Table II.

TABLE I

ANALYSIS OF *C. sapidus*

Description of Condition	Sex	Solids Percentage	Nitrogen Percentage in Solids	Glycogen Percentage in Solids	Sugar Percentage in Solids	Glycogen / Nitrogen	Sugar / Nitrogen
Hepato-Pancreas							
Hard crab	M	4.8	8	<0.02	1.16	.....	0.145
	F	4.1	8.7	<0.02	1.27	.....	0.146
Approaching molt	M	3.15	9.1	0.10	0.13	0.011	0.014
	F	6.6	6.7	<0.02	1.58	.....	0.236
To molt in few hours	M	2.95	11.2	<0.02	2.34	.....	0.209
	F	6.9	6.5	<0.02	1.48	.....	0.228
In act of molting	M	1.7	7.3	0.04	1.56	0.006	0.214
	F			(Missing)			
Soft	M	1.96	10.8	0.04	4.44	0.004	0.411
	F	1.50	7.7	0.05	3.47	0.006	0.451
Whole Crab Except Hepato-Pancreas and Digestive Tract							
Hard crab	M	4.1	12.4	<0.02	1.41	.....	0.114
	F	6.7	7	<0.02	1.43	.....	0.204
Approaching molt	M	5.8	10.3	3.4	0.15	0.33	0.015
	F	7.1	9	<0.02	1.69	.....	0.188
To molt in few hours	M	6.5	9.2	<0.02	1.28	.....	0.139
	F	7.9	9.8	<0.02	1.27	.....	0.13
In act of molting	M	3.7	7.4	<0.02	1.45	.....	0.196
	F	3	11.5	<0.02	0.96	.....	0.084
Soft	M	3.3	12.1	<0.02	2.16	.....	0.178
	F	1.9	8.9	<0.02	2.1	.....	0.236

Claude Bernard (1877, 1879) and Vitzou (1882) found the glycogen content of the crab hepato-pancreas to be very high immediately before molting, and Schönborn (1910, p. 536) found 1.0, 0.85, and 2.2 per cent in *Carcinus maenas* before molting and 0.4 per cent

(p. 539) immediately after the molt. Hoet and Kerridge (1926, p. 117) found 0.55 per cent in hard *Cancer pagurus* and less than 0.03 per cent in soft specimens.

In none of these cases would the conversion of the glycogen to sugar sufficiently raise the osmotic pressure to account for the freezing-point depressions observed. However, if a glycogen content of

TABLE II*

Condition	Hepato-Pancreas		Remainder of Body		Total	
	Sugar	Glycogen	Sugar	Glycogen	Sugar	Glycogen
Hard	None	0.325	0.064	0.017	0.062	0.028
Approaching molt	None	None	None	0.115	None	0.110
Three-quarters molted	.....	.....	.....	.....	Trace	0.065

* Results in percentage of wet weights.

0.55 per cent by wet weight were converted to hexose diphosphate ester from hexose, and phosphate from some insoluble calcium-phosphate compound, a not impossible assumption, it is easy to calculate that an increased freezing-point depression of a third of a degree could be obtained when the hexose diphosphate was converted to lactic acid, hexose, and B_2HPO_4, according to Meyerhof's scheme.

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THE NATURE OF THE APPARENT GEOTROPISM OF YOUNG RATS

(Five figures)

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THE aim of work on animal conduct is to reduce responses and the factors controlling them to mathematically predictable terms. The tropism, which is generally accepted as being an unconditioned machine-like reaction of the organism to a field of force, permits of such analysis. Recently Crozier and his students have attempted to apply the theory of tropisms to mammals, i.e., the young rat. In one paper (Crozier and Pincus, 1926*a*) appears the statement that "they [tropisms] are as definite and predictable [in the rat] as in the most favorable instances among invertebrates"; and also that if white rats are "allowed to creep on a fine meshed wire grid, orientation is always upward." Crozier and Pincus (1926*b*) report that white rats 13 and 14 days old ascend inclined surfaces in such manner that the average angle of orientation (θ) is proportional to the logarithm of the component of gravity parallel to the inclined surface (see Fig. 1). Crozier and his students have subsequently published elaborate repetitions of this view. They reported an equilibration (Crozier and Pincus, 1927*a*) between conflicting "phototropic" and "geotropic" responses in the rat, such that the ratio $\frac{\log \text{intensity}}{\log \sin \alpha}$ is a constant; and consequently they claim a further confirmation of the tropistic interpretation. They state that the equation $\theta = K \log \sin \alpha$ is applicable in the "geotropic" responses of roof rats, *Rattus rattus* (Crozier and Pincus, 1927*b*) and mice (Crozier and Oxnard, 1927). Assuming the response to be a tropism, Pincus (1927) has measured the speed of upward creeping of young rats. Now if, as Crozier and his co-workers assume, young rats show negative geotropism, one should find rats with eyes yet unopened trying to climb out of the nest or up the sides of the cage. This in no case have we been able to observe. Consequently the

validity of the geotropic interpretation of the reactions of young rats appears questionable.

In order to obtain clear evidence on these points I have carried out experiments to determine the nature of the response of the young rat to the inclination of the plane on which it stands. In the last analysis, the rat, no doubt, behaves as a machine; but it is quite probable that the complexity of behavior of such an organism renders a tropistic analysis of its activities comparatively difficult. In their chief paper Crozier and Pincus (1926*b*) base their conclusions on observations made on but two selected individuals. They nowhere state what constitutes a trial. The results are therefore hardly

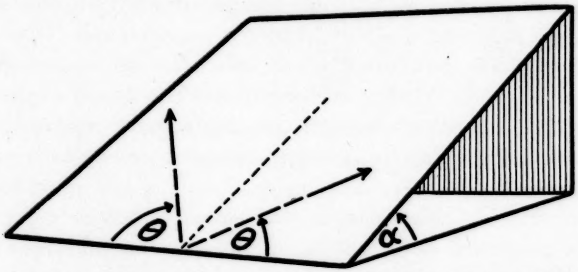

FIG. 1.—Diagram showing the meaning of symbols used to express angles of inclination of the creeping surface to the horizontal (α), and angles of orientation of the rat's path on the plane (θ). (Same notation as used by Crozier and Pincus.)

susceptible of generalized treatment. In my experiments no selection of material was made—all the individuals in each litter were used.

The apparatus consisted of a shallow box having a floor 24×32 inches in size which could be inclined by means of wooden props to the angles of 30°, 40°, 50°, 60°, 70°, and 80° from the horizontal. The floor was painted with co-ordinate lines 1 inch apart, over which a $\frac{1}{8}$-inch mesh screen (fruit-drying cloth) was stretched. The reactions of the rats were tried out under both daylight and red-light illumination; but since there appeared no variation in the responses under the two conditions, the tests were conducted for the most part under ordinary day and night lighting conditions. Room temperature was always maintained between 21° and 24° C. Records of trails were made by placing gum markers on the grid behind the animals as

they progressed. No trail less than 5 inches was used in the calculations. At the start the animals were always placed head upward.

EXPERIMENTAL

A litter of six albino rats was tested 7 days after birth. After being placed on the screen, an individual remained in position for a few seconds before moving about at random. At this age the animal was too weak to ascend at an inclination α of 30° from the horizontal. On the eighth and ninth days the animals were able to climb somewhat at the lower values of α, and they also became progressively more active. Attempts to climb downward or toward the sides resulted in slipping and falling—the rats usually fell once or more before making successful climbs at the greater angles, even on the twelfth day. On the tenth day and thereafter attempts to climb up predominated. On about the eleventh day the animals were able to cling to the screen as long as their climbing was upward. In fact they could cling while going in any direction so long as their bodies were oriented upward. Creeping to the sides and downward was accomplished in this manner. Occasional sidewise creeping responses continued until the end of the experimentation with this litter. The great majority of runs, however, were upward.

These experiments indicated that upward creeping either is a learned response, and not a tropism, or else is a tropism which appears several days after birth when the somatic musculature is more fully developed, i.e., just before 14 days. In order to decide this point, a fresh litter of 13-day-old rats containing seven individuals was tested. Rats at this age are well able to climb the screen at angles of inclination as high as 80° and do not fall when they are facing the top. The testing was run through the thirteenth, fourteenth, and most of the fifteenth day until the time arrived when an eye of one of the rats began to open.

This second set of experiments was planned with a view to determine whether or not upward creeping is a conditioned response. If the reaction should increase in accuracy with repeated trials, it would be necessary to conclude that memory and not a tropism is the determining variable in the response. The dimensions of the creeping surface were changed to 24×24 inches. A "trial" was made

in the following way: The subject was placed in the center with its head toward the top and was left on the surface until it reached the top, the bottom, or one of the sides, after which it was returned to the nest; if it fell before reaching a boundary, it was placed on the grid again. A rest interval of at least 15 minutes was given between any two trials. The time for each trial was taken with a stop watch. Errors consisted of falls or crawling to one of the sides or the bottom. (The latter is justifiable on the grounds that the perfected response is upward creeping.) All the attempts to creep in wrong directions were far too numerous to be counted.

In order to construct a comparable time curve, each determination on the curve was made from the sum of thirty-five readings, or trials—the time between being placed on the grid and reaching a boundary for each of the seven subjects at each of the angles of inclination, 40°, 50°, 60°, 70°, and 80°. There were eighteen such determinations. Each determination is designated as a Σ trial. The 30° angle was omitted because the force due to gravity which seems to impel the animals to activity is disproportionately less than it is at the greater inclinations, as will be discussed farther on. The logarithms of the time determinations (Σ trials), each based on thirty-five readings, were plotted as ordinates; and the numbers of the determinations were plotted as abscissae, producing the curve in Figure 2.

It will be noticed that the curve in Figure 2 follows a learning curve. There is a fairly constant decrease in time for the successive Σ trials—speed of the reaction increases with practice, indicating progressive decrease in errors. The rise at Σ trial 9 is due to a lapse of 10 hours between the fourteenth and fifteenth days, and another rest of 4 hours is responsible for the rise at Σ trial 12. This means that a considerable lapse of time without trials resulted in a forgetting and consequently a lengthening of time for a successful trial. The other slight irregularities are probably due to such undetermined extraneous factors as increased activity aroused by contractions of the stomach walls, lengthened rest intervals necessary for feeding, etc. The figures in Table I, therefore, show that as practice continues, precision of the response increases. After Σ trial 9 every rat crept upward. The facts prove that upward creeping in the

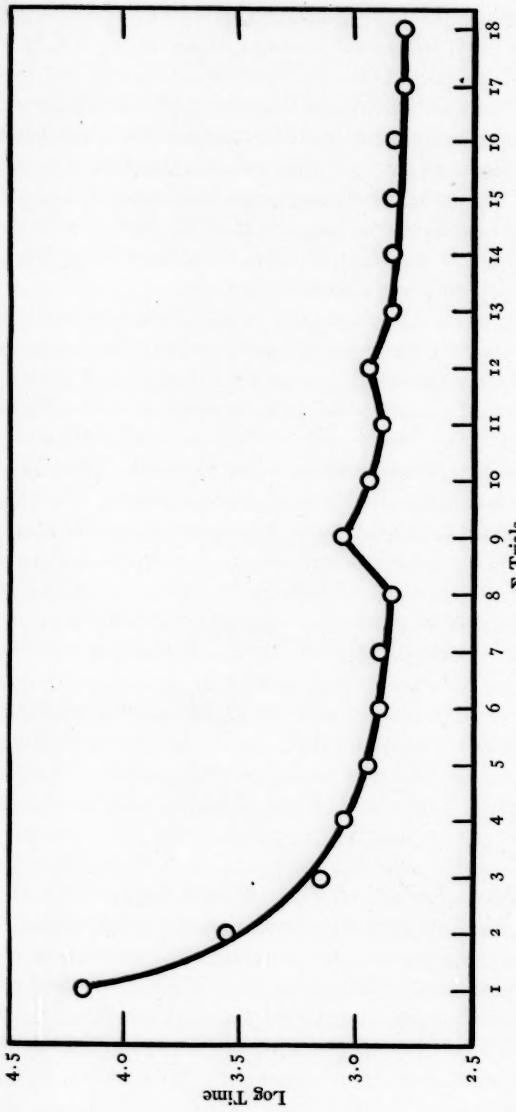

FIG. 2.—When young rats were placed on the inclined creeping surface, the time required for them to reach a boundary was reduced as the number of trials increased. A Σ trial represents thirty-five trials—one for each of the seven rats at 40°, 50°, 60°, 70°, and 80° a. One individual trial is consummated when a rat reaches any boundary. The log of the total time for each Σ trial is plotted on the ordinates. The rises at Σ trials 9 and 12 are due to relatively long time intervals between them and the preceding Σ trials, respectively, allowing a forgetting.

albino rat is the result of learning, and consequently is in no sense a tropism.

Still further evidence on this point was given by tests made with seven untrained 21-day-old rats whose eyes had been covered with collodion before signs of opening were manifest. They showed random movements climbing in directions other than upward. They went up seventy-four times, to the sides forty-seven times, and to the bottom twenty-nine times in thirty trials at 40°, 50°, 60°, 70°, and 80° α. At this age the muscular apparatus is more fully developed and the animal has more control of its legs, so that it is

TABLE I

Σ trial.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Wrong side.....	14	4	2	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Falls.....	90	16	1	4	2	2	5	1	0	1	0	0	1	0	0	0	0	0
Time (sec.).....	21,232	4,300	2,125	1,184	771	618	620	496	1,228	810	563	892	537	492	466	525	371	420
Age (days).....	13	14							15									

When young rats are first placed on the inclined surface, some of them, very laboriously, creep toward the sides and the bottom. They also fall at times when attempting to creep in a direction other than upward. By means of practice they learn to follow the path by which a boundary can be reached most easily, and without any falls. A Σ trial represents thirty-five trials—one for each of the seven rats at the five values of α.

enabled to climb in any direction without slipping, but is most adept at creeping upward. Such behavior shows conclusively that training (i.e., conditioned reflexes) is at the basis of non-varying upward creeping of young rats. If the phenomenon were a tropism, the animals would invariably creep upward without any previous training, and furthermore, training would not affect the precision of the response. But this, as I have shown, is contrary to fact.

The question arises as to why the animals climb up instead of down or to the sides, and why they creep at all when placed on an inclined surface. The latter is apparently due to geokinesis or excitation by the gravitational stimulus. Observations show that the rat's muscular apparatus is not well enough developed at 13 days to enable it to crawl with readiness toward the sides or toward the bottom; and also, due to the arrangement of the claws, down-

ward creeping results in slipping, while in upward creeping the claws hold most easily to the wires of the grid. Whenever the 14-day rats attempted to climb down or toward the sides, their hind feet would slip or else they would lose grip entirely; more effort was required to reach a side or the bottom. Consequently, inhibitions were built up against slipping, with the result that upward creeping

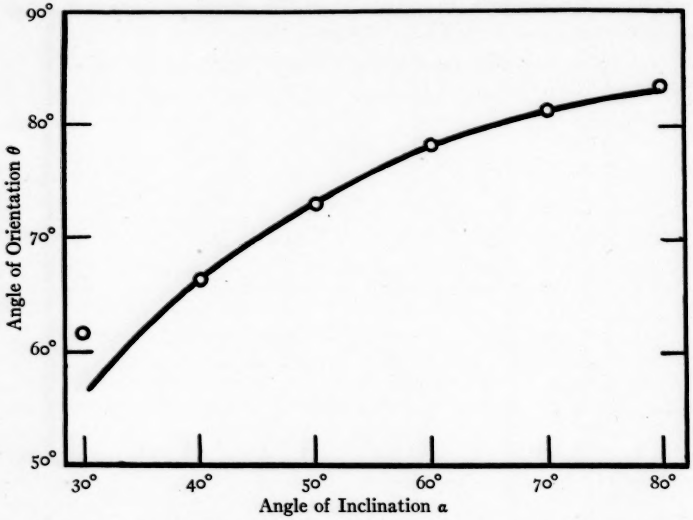

FIG. 3.—The mean angle of upward orientation (θ) increases as the inclination of the creeping surface (α) becomes greater. For values of α, the θ's are as follows: 30°–61.3°, 40°–66.3°, 50°–73°, 60°–78°, 70°–81.2°, and 80°–83.3°. Each point is taken from the average of from 115 to 188 runs 5 inches or more in length—938 in all.

was more and more used, and therefore became the conditioned response.

It is also apparent that physical forces independent of the animal's nervous system play a part in determining the nature of the response; for if, instead of a rat, a rectangular block of wood long enough to prevent its tipping on end, is placed on an inclined surface (of maximum friction) there is an angle θ below which, within limits, the block will roll over. The greater the angle of inclination of the surface, the greater will be the critical angle θ for the block; it is such that $\cos \theta = \frac{w}{h} \cot \alpha$, where w is the width of the

block and h the height. Young rats lose their grip when they do not orient upward. Therefore it is reasonable to suppose that critical angles obtain for them as for a block of wood, if we take into consideration the fact that their feeble claws will not sustain much of their body weight, making it impossible for them to cling to the screen when the force pulling the claws on one side of the body—the upper side—exceeds a certain value. It is obvious, however,

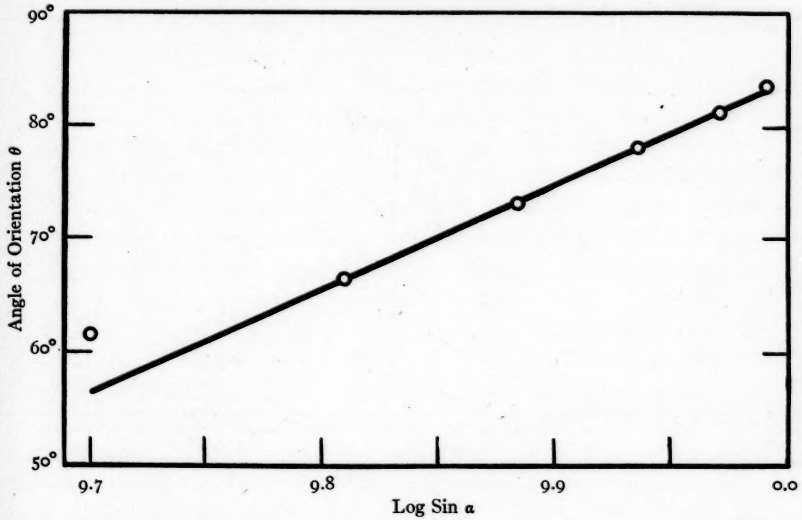

FIG. 4.—The average angle of orientation (θ) is proportional to log sin (α), the angle of inclination of the creeping surface—around $\alpha = 40°$ and above. The six points taken together represent 938 readings.

that the critical angles for the rats could not be directly compared with those for an inanimate object of the same dimensions, inasmuch as the rats are continually changing shape and have some ability to cling. Now, Figure 3 represents the mean θ's for the rats plotted against values of α, which shows that they climbed more directly upward as the inclinations of the plane became greater. This is readily accountable in the fact that there are fewer directions open to proficient creeping at the greater inclinations.

The average of the angles of orientation, as based on straight-line runs of 5 inches or more, shows a relationship to the component

of the gravitational pull parallel to the inclined plane, similar to that found by Crozier and his co-workers. Figure 4 shows that the mean values of θ on either side of the vertical, plotted against log sin α, approximate a straight line, except where $\alpha = 30°$. Since the critical angle approaches the vertical as the inclinations are increased, it is logical to expect the average of the runs to approach the vertical also. This they do. Furthermore, the average values of

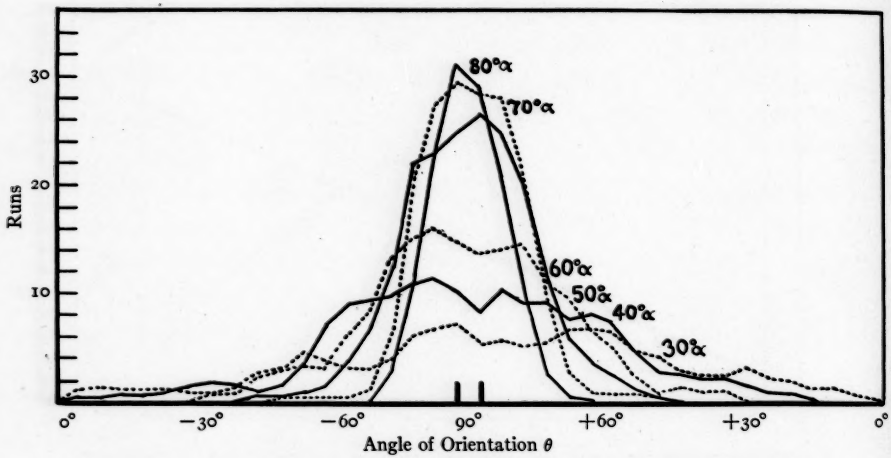

FIG. 5.—These curves represent distributions of runs both to the left and to the right, at each of the six angles of inclination of the creeping surface. Each curve was smoothed with a moving average of 3. Each approximates, roughly, a normal frequency curve. (It may be noted that they are multimodal, perhaps due to the small number of determinations.)

θ are not important to an understanding of behavior, for the reason that the averages do not represent the angles near which the greatest numbers of runs were made. After plotting distributions of runs in terms of θ for each of the six values of α, the curves were found to be skewed toward the vertical in each instance. Distributions were then plotted in terms of plus and minus 90°; that is, 90° was taken as the midpoint on the base line with right-side runs plotted to the right of 90° and left-side runs plotted to the left. All six curves resemble an average distribution curve with the highest mode near 90° (Fig. 5).

DISCUSSION

After having finished my experiments, I discovered a paper by Hunter (1927) in which that author obtained results similar to mine in respect to distributions and frequent failures of the animals to creep upward. He found a straight-line relationship between the mean values of θ and log sin α but shows this to be fictitious for the reason that the means showed little correspondence to the medians. We should expect such curves to be bimodal if the average of θ is important to the analysis of the behavior of rats when acted upon by gravitational forces. But this average becomes merely a measure of variability—the average deviation of runs from the line perpendicular to the base line; the deviations becoming less as α is increased. Hunter's distribution curves and also my own show flattenings correlated with decreasing angles of inclination. This might be expected since the critical angles on either side of 90° become farther apart as the inclinations of the creeping surface are decreased, because they restrict less the number of directions open to the rat.

In the present experiments a rectilinear relationship obtained only for values of α greater than 30° (Fig. 4). The point representing θ log sin 30° deviates considerably from the line passing through the other five points. There is no critical angle for the rats, apparently, at this inclination. All directions would be open to the rat in such a situation. Upward creeping here, would then be merely a conditioned response built up through creeping at the greater inclines.

The claims of Crozier and his students, as set forth in a series of articles dealing with geotropism in mammals, would be very stimulating to additional work on animal conduct if these claims could be confirmed. Unfortunately these workers apparently have been the victims of self-deception. In the light of my results, it may reasonably be supposed that in Crozier's experiments the individuals which were selected from a litter formed conditioned reflexes more readily than the average, or they may have learned to creep upward quicker because of other unobserved factors. In his experiments many responses on the inclined surfaces which should have been taken into account may have been overlooked.

My experiments on three whole litters of white rats—twenty individuals in all—show that consistent upward creeping of the

rat is the result of two factors, namely, geokinesis and a conditioned response, so that, as a result of training, movement is upward on the inclined grid. Since the orientation of untrained individuals is never machine-like and upward, the conclusion is clear that the mechanism of orientation is not tropistic in the accepted sense of the word. Unquestionably all locomotor movements in young rats are at bottom mechanistic, but the mechanism is complex and not simple. An assumption such as Crozier's, that the response of young rats to gravity is a simple tropism, therefore only deceives by a false simplicity.

SUMMARY

After several trials, young albino rats with unopened eyes ascend inclined surfaces on which a good footing can be obtained. Contrary to the interpretations given by Crozier and his students, the experiments show that this response is the result of a conditioned response —not a tropism. The increased movement of the young rat when placed on an inclined plane is probably the result of muscle tension in the animal's body. It seems reasonable to assume that the animal creeps upward because inhibitions are developed against downward and sidewise creeping as a result of purely physical and structural factors such as the arrangement of its claws and legs, shifting of the center of gravity within its body, etc., which tend to prevent it from moving in any direction other than upward.[1]

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[1] The writer wishes to express his thanks to Dr. A. R. Moore for suggesting the problem and for criticism of the manuscript.

INDUCED ENCYSTMENT AND EXCYSTMENT IN EUPLOTES TAYLORI, SP. NOV.

(Three plates)

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INTRODUCTION

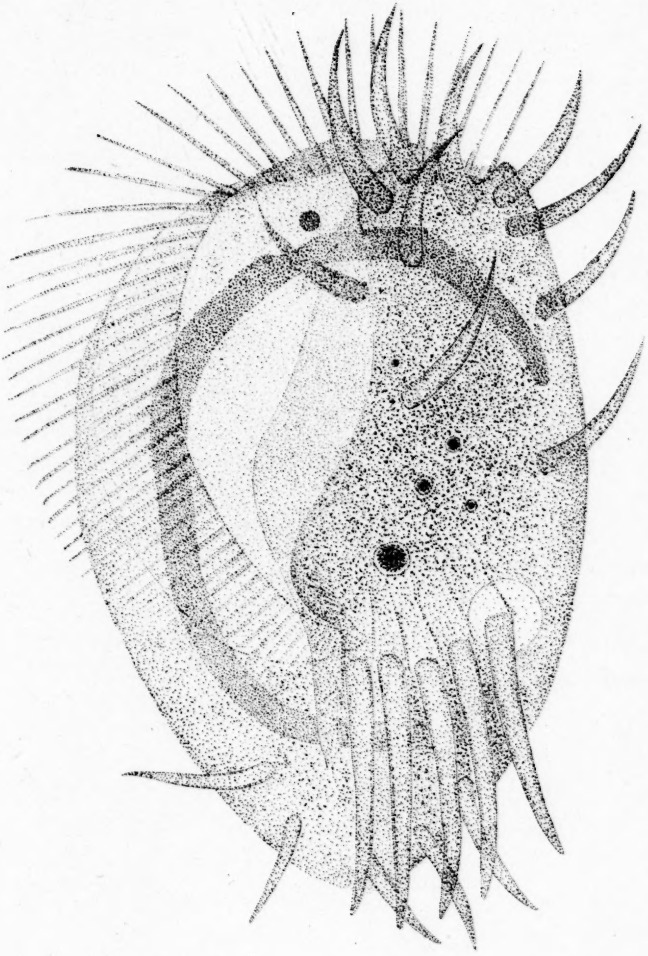
ENCYSTMENT has been known to occur in the genus *Euplotes* since 1854, when A. Schneider briefly described this process in *E. charon*. The following year Stein described the cysts of the same species and also observed empty cyst walls. Since this early time, cysts for two other species have been reported —*E. harpa* and *E. longipes*. Mélant (1922) obtained cysts of *E. harpa* experimentally by increasing the salt content, by adding excessive amounts of any one of the common compounds found in sea water, or by addition of sugar, and concludes that the osmotic pressure of the liquid is an exciting agent for the formation of cysts. He does not describe either encystment or excystment. Stolp (1924) found occurrence of encystment in *E. longipes* and obtained a complete line from a single excysted animal. However, he does not mention that he observed either process. In the same laboratory, Klee (1926), studying the life-cycle of the above species, brought about encystment by allowing cultures to stand with infrequent changes of sea water and by increasing the temperature. She states, however, that she was unable to cause excystment in a single case (p. 351).

Euplotes taylori, sp. nov. (Plate I, Fig. 1), was discovered in material collected from a pond in San Francisco Bay. This pond is so situated that it receives both fresh water from winter rains and salt water from the Bay. Thus its salinity varies greatly from time to time. Collections made in December, 1926, when first examined contained a species of *Euplotes;* but the form in question was not encountered until the culture had stood in the laboratory for several weeks and fresh water had been added to replace that lost by

PLATE I

FIG. 1.—*Euplotes taylori*, sp. nov., ventral aspect. Camera lucida drawing of living animal. ×465.

PLATE I



evaporation. At this time, without due warning it became extremely numerous. The sudden appearance of such vast numbers cannot reasonably be attributed alone to the presence of a few forms in the beginning and their multiplication by division. The discovery of cyst formation in this species, made in March, 1927, readily explains the phenomenon.

As might have been expected, there was a decline in the number of *Euplotes* after a few days. Then, in order to provide concentrated cultures for a taxonomic study, small quantities of the medium were set aside in moist chambers until the organisms became numerous. In this manner a group of four cysts was discovered in a dish in which evaporation had gone on to such an extent that only a film of water remained. Fresh culture medium was added with the result that after a time each cyst appeared double. To discover what had actually happened, the high-power dry was used effectively as a water immersion objective and thus the details of four excystments were observed. Numerous empty cyst walls and many free organisms were later found in the same dish. Within a few days a complete encystment was observed, followed immediately by induced excystment; and since this time both processes have been repeated countless numbers of times, and at will.

ACKNOWLEDGMENTS

This study has been made possible under the very helpful direction of Professor C. V. Taylor, of Stanford University. In appreciation of the contributions made by Dr. Taylor to the literature of the genus *Euplotes*, I have pleasure in naming this new species in his honor.

My thanks are also due to Mr. James E. Lynch of Berkeley, California, for sending the material in which *Euplotes taylori* was discovered.

EUPLOTES TAYLORI SP. NOV.

Diagnosis.—Body oval; anteriorly truncate, with projecting oral lip; posteriorly bluntly pointed; left margin curved more than right. Pellicle convex, flattened posteriorly, having eight longitudinal ridges. Ventral surface flattened; anterior margin with two denticulations—left very shallow, right deep, forming prominent tooth-

like process. Cytostome two-thirds to three-fourths length of body. Cirri: ten frontal, five anal, six (or four to six) marginal. Contractile vacuole on right ventral side, beneath outside anal cirrus. Macronucleus C-shaped; micronucleus spherical, situated outside upper left bend of macronucleus. Length, 72–100 microns; greatest width, 50–60 microns. Habitat, salt or brackish water. Locality, San Francisco Bay.

E. taylori resembles *E. harpa* in having a carapace with longitudinal ridges and toothlike process at right anterior ventral margin, but conspicuously differs from it in contour and length of body, and number of marginal cirri.

TECHNIQUE

Culture.—The organisms used in this study were the descendants of those discovered in the material collected in December, 1926. The method of culturing is as follows: A stock supply of brackish water in which unicellular algae and small ciliates and Flagellates thrive, is kept in the laboratory. This medium is pipetted into Syracuse watch glasses, diluted with 2 or 3 cc. of distilled water, and seeded with *Euplotes* from a previous culture. The watch glasses are then placed in moist chambers. New cultures are started from time to time. It has been found that if encystment is induced in such a small culture, followed by excystment, the *Euplotes* become exceedingly numerous within 2 or 3 days.

Methods of preparation.—In order to make permanent mounts, attempts were made to transfer cysts to slides or coverslips by means of a micro-mouth-pipette. This proved exceedingly difficult, as was found also to be the case by Klee (1926). Cysts adhere to glass and are often injured when finally removed. Cysts formed on the surface film were successfully carried over, but even with application of albumen fixative became detached from the slide at any time during the staining process and so were easily lost. A few specimens were completed in this way with fairly good results.

The adhesive quality of the cyst membrane suggested the possibility of a better method, i.e., of cyst formation directly on slide or coverslip. To this end from twenty to thirty animals were transferred within a drop of culture medium to a slide (or coverslip) and then placed in a moist chamber. Within 1–10 hours, and even less

time, total encystments occurred. The temperature at which the best results were obtained in a minimum of time was found to be about 22° C. If older cysts were desired, the coverslip was inverted on a depression slide (in which an extremely minute drop of water had been placed) and the edges sealed with vaseline. The advantages of this method are the ease with which accurate data may be kept and the definite location of cysts on the slide in stained preparations.

The first steps of the staining process were made under a low power binocular. The remaining water on the cysts was drawn off with a pipette, and hot Schaudinn's fluid (without acetic acid) immediately applied. (Bouin's fluid also gives good results.) This fluid was drawn off with filter paper, and a second application of the hot Schaudinn's made. After a few minutes, cysts were washed in 50 per cent alcohol, followed by iodine solution, and again by 50 per cent alcohol. The slides or coverslips could now be transferred to staining jars for completion of the staining process without danger of cysts becoming detached. Heidenhain's iron-haematoxylin was used for staining.

The method of obtaining cysts for staining purposes proved to be an excellent means of studying the details of both encystments and excystments in living condition. The coverslip with the drop of water containing *Euplotes* was placed in a moist chamber until signs of beginning encystment were noted. The coverslip was then inverted on a depression slide and the edges sealed with vaseline, as mentioned above. The slide could then be placed under the microscope without danger of rapid evaporation. Within the hanging drop the complete process of encystment could be noted, and under any magnification, including oil immersion. To study the process of excystment, the coverslip was removed, a drop of fresh culture medium (or tap or distilled water) was added, and the coverslip replaced.

ENCYSTMENT

Under the low-power binocular, typical cysts of *E. taylori* appear as minute drops of water. With higher magnification they are found to be more or less ellipsoidal in shape, with a diameter varying from about 40 to 55 microns, and approximately 20 to 28 microns in

depth. Cysts may be found singly, but more often they appear in groups (Plate II, Figs. *2a, b, c*). The groups were first observed while encystments were taking place within a Syracuse watch glass. They have since been very commonly found also when encystments were induced within drops of the culture medium. It might be supposed that the occurrence of these groups of cysts within drops was quite incidental since the animals would have a tendency to remain in the spot of least pressure, i.e., in the center. However, certain facts have been noted which cause one to wonder if there may not be some significance in this procedure. It was first observed by Dr. Taylor that in several cases a free organism upon coming in contact with a cyst would immediately begin encystment. Cysts of an unknown species of *Diophrys*, which were induced by the method herein described, also have been found by the author to group themselves in a similar way.

The first apparent indication of oncoming encystment is decreased motility. In individuals which are gorged with food, this change is accompanied by the extrusion of some food through the anal aperature. This extrusion was first noticed in encystments under a coverslip, and for this reason its cause was attributed to pressure, but was found later to occur also in drops of water of considerable depth. The animals now creep about apparently with less and less facility; the cirri tend to adhere to the glass; and finally the animals become fixed to the slide (Plate II, Fig. 3). The cirri are often curled over at their tips, or they become broken up into their component cilia. The membranelles and marginal cirri, however, beat vigorously at this stage. Later the movement of the membranelles is confined to the region near and within the cytopharynx, where this movement may continue intermittently even as long as from 10 to 15 minutes. The membranelles, anteriorly, become drawn closer and closer to the body until they are no longer visible. In some cases, as they are thus being drawn to the body, the membranelles break up into their component cilia, which soon merge into one another and so lose their individual identity. At about the same time the posterior end of the body becomes gradually rounded (Plate II, Figs. 4, 5, 6). The more fluid endoplasm moves centripetally and over the cytostomal region, apparently in convergence

of a peripheral contraction, whereupon the cyst comes to assume, chiefly on the dorsal side, a rounded contour. The anal and marginal cirri at first project from the right side. Gradually these also have become drawn to the body wall. The contractile vacuole in the greater number of cases observed is functional during the entire foregoing process.

By the time the animal assumes this rounded form, a cyst membrane is present. The membranelles, if present, show a vibrating movement, and this may continue for a considerable time. Sooner or later, however, all movement ceases, and the membranelles disappear from view.

The cyst now has a granular appearance (Plate II, Fig. 7); the central region is coarsely granular and contains food particles. Deeper within the cyst, the membranelle region can be distinguished by its homogeneous appearance. A vacuole may or may not be in evidence. During a period of 7 days in which a group of eleven cysts were under observation, vacuoles appeared in all at intervals. These cysts, however, were kept within a sealed depression slide and thus the external conditions remained fairly constant. Cysts within unsealed watch glasses 2 weeks old (covered with about $\frac{1}{4}$ inch of water) have no vacuoles.

How long *Euplotes* can remain in this quiescent stage is not known. Klee (1926) states that cysts of *E. longipes* were observed in living condition from 20 to 25 days. Excystment has been induced in cysts of *E. taylori* 4 weeks old. There is apparently no definite period of encystment necessary (beyond 15 or 20 minutes), because it is possible to induce excystment immediately following encystment, and therefore excystment must depend upon a change in the surrounding medium. When a decided change in the concentration of the medium is suddenly made, the protoplasm sometimes flows from the cyst wall but soon disintegrates.

The cyst is protective against a sudden change in the culture medium. When a large drop of tap or distilled water is added to a drop containing both free-living and encysted forms, the medium thus being diluted to about one-half, the free-living forms invariably disintegrate, while the encysted ones emerge from their walls in due time and thrive thereafter. Excystment in *E. taylori* has not

PLATE II

FIGS. 2*a*, *b*, *c*.—Photo-micrograph of prepared slide. Three cyst groups formed within one drop of culture medium. ×143.

FIGS. 3–7.—Stages of encystment. Camera-lucida drawings of living organisms. ×465.

FIGS. 8–12.—Stages of excystment. Camera-lucida drawings of living organisms. ×465.

FIG. 13.—Empty cyst wall.

PLATE II

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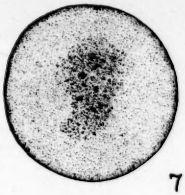

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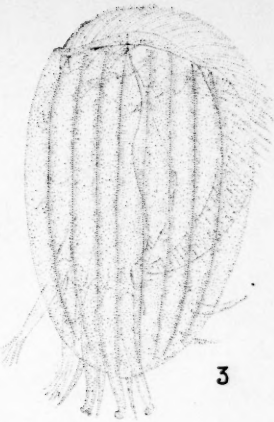

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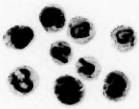

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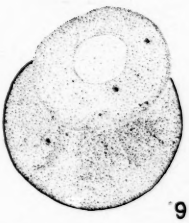

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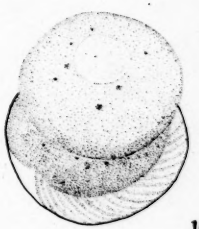

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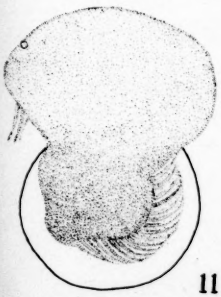

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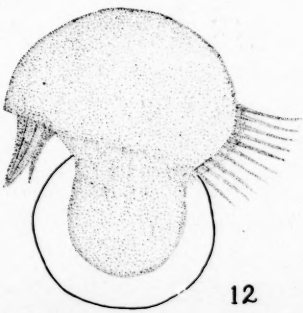

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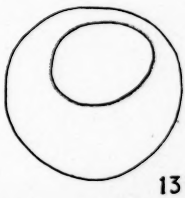

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been produced after complete drying. It is probable, however, that the drying process was too rapid. Under natural conditions cysts, because of the sticky nature of their early membrane, would be covered with débris in the pond, and this would prevent rapid drying. Excystment has been induced when crystals have already formed over the entire surface. In one such case, the time for excystment was prolonged to 35 minutes; the movement of membranelles and cirri was feeble; and the organism came out slowly. The resulting *Euplotes* was highly vacuolated, but within 2 days assumed a normal appearance.

EXCYSTMENT

The first striking change in the cyst toward excystment is that of the reappearance of the contractile vacuole (Plate II, Fig. 8). Next, a hyaline semicircle (Plate II, Fig. 8) suggestive of the membranelle region is noted. In cysts about 12 hours old the wall expands from 6 to 10 microns. The contractile vacuole gradually increases in size; and after contracting once, sometimes twice, or not at all, in which case it becomes extremely large, suddenly the cyst wall breaks (Plate II, Fig. 9). The protoplasm can be seen streaming to the outside, where it forms a sphere; and at a stage when about half of the cyst contents protrudes (Plate II, Fig. 10), ciliary movement is noted on the portion still within the cyst wall. This movement aids in pushing more of the contents to the outside. Almost instantaneously a part of the membranelles and cirri appear just outside the wall (Plate II, Figs. 11, 12). Now the movements within the wall become extremely vigorous; and in a very brief time the animal, apparently full-fledged, is out and moving freely about in the water. As it leaves the wall, one is impressed with the large size of the organism as compared with the space within the cyst wall. A considerable quantity of water is undoubtedly absorbed during this process. Although the *Euplotes* may be humped or variously dented at first, it soon (3 minutes in one case) assumes the normal appearance and moves about rapidly. The contractile vacuole in some cases is two or three times its natural size, but after the first contraction functions normally. Within an hour or more following excystment the animals become gorged with food. This is particularly noticeable when unicellular algae are present.

The entire process from the time of the addition of fresh water to the fully formed *Euplotes* leaving the cyst wall can be completed in less than 10 minutes. This, however, is true only under certain conditions and when cysts have been quite recently formed. Older cysts possess rigid walls; and this fact prolongs, in part at least, the period preceding the breaking of the cyst wall. In one instance of a cyst 10 hours old, it took 12 minutes for the break to occur; and when it did so, the protoplasm came out with great force. Such a wall does not collapse but remains for some time as a smooth, transparent globe with the large opening through which the animal escaped (Plate II, Fig. 13). A cyst wall of this nature was described by Schneider (1854) and Klee (1926). The wall of cysts 15 to 30 minutes old, upon induced encystment, breaks easily and the protoplasm flows out slowly. Thereupon, when the animal has become fully formed, nothing remains of the wall except an amorphous mass of gluelike substance. The glutinous nature of the wall is indicated by the fact that the cirri of the newly excysted organism often adhere to it; and in one such case the animal could not escape for more than an hour, although it made efforts to free itself.

Uninduced excystments have been observed. This natural excystment appears to be the same as that of the induced. Slides on which total encystments had occurred about six o'clock at night contained only free-swimming organisms at eight o'clock the following morning. By noon of the same day total encystments had again occurred in many cases. After the second total encystment, and sometimes after the first, both free-living animals and cysts were to be found. Why individuals which at first encysted at approximately the same time should next exhibit differences in this respect under similar conditions may be attributed to a number of causes. Conditions in a drop of water on a slide placed within a moist chamber would not, of course, remain at all constant. Changes in temperature, quantity of food present, concentration of salt content, and waste products become factors of importance. If the slide is removed from a large moist chamber to a small one (such as a depression slide provides) and thus the evaporation is reduced to a minimum, animals remain in the encysted state for a longer period.

It is of interest to note here that two cysts were found which did

not conform to the usual shape or size but whose cyst wall resembled closely that of the pellicle of the differentiated animal. Within the cyst wall there was no suggestion of membranelles or cirri, but the whole presented a granular appearance throughout. To test its living condition, excystment was induced. In the one case a vacuole appeared. At about the same time a transparent streak suggestive of a cytostome was noted. The vacuole contracted three times, and when the fourth had reappeared and had increased to an abnormally large size, a break occurred in the cyst wall. The changes which take place during excystment were even more striking than that of other cysts because of the comparatively larger space within the cyst wall. In the second case, the cyst was formed beneath a coverslip. When fresh water was added, rotation of the contents occurred; the micronucleus could be followed clearly in its movement. Later, membranelles appeared and excystment took place.

These questions may be asked: Does encystment continue when once the process has been initiated? Or, when fresh water is added, is the process interrupted at any stage and reversed? If fresh water is added when the organisms have become almost motionless, some resume movement, others disintegrate, and still others continue toward encystment. It appears then that at a particular time this process continues to completion in spite of the change in the surrounding medium to that favorable to excystment. It is interesting to note that in some cases observed, the distal ends of membranelles and cirri may disintegrate upon the addition of water, yet encystment continues. The droplets of protoplasm formed from the disintegrating membranelles are drawn to the body in some cases; and when excystment is later induced, the organism emerges with complete membranelles.

ABNORMAL CYSTS

It must be stressed that gradual evaporation of the culture medium is necessary to produce normal cysts. This is true especially in cyst formation on slides. Here 21°–22° C. within a moist chamber brought about a condition which gave the best results, i.e., total encystment. Good results were also obtained at lower temperatures.

A rate above the maximum evaporation can be determined by

PLATE III

FIGS. 14–20.—Camera-lucida drawings. Heidenhain's iron-haematoxylin stain. ×1230.

FIG. 14.—Cyst, early stage.

FIG. 15.—Cyst showing wall.

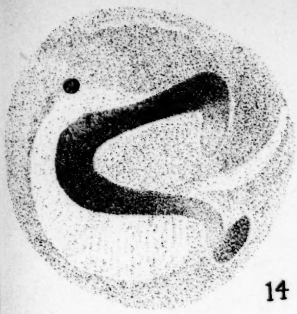
FIGS. 16, 17.—Cysts, late stage.

FIG. 18.—Stage in binary fission. Micronucleus not evident.

FIG. 19.—Stage in conjugation. Detail in micronuclei not shown.

FIG. 20.—Excystment. Upper portion still within cyst wall.

PLATE III



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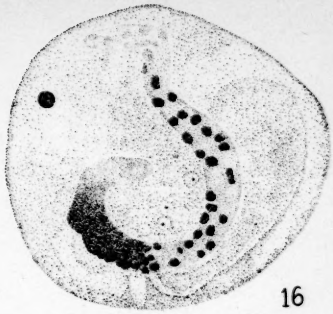

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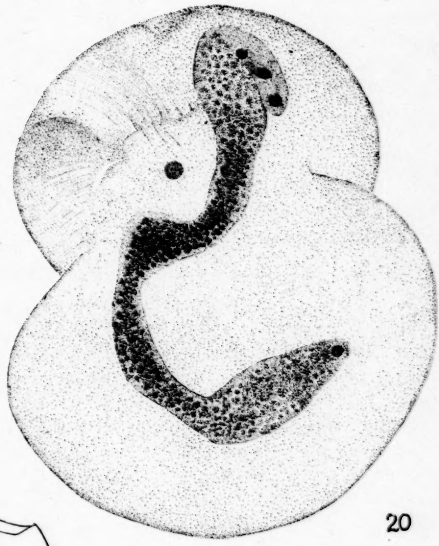

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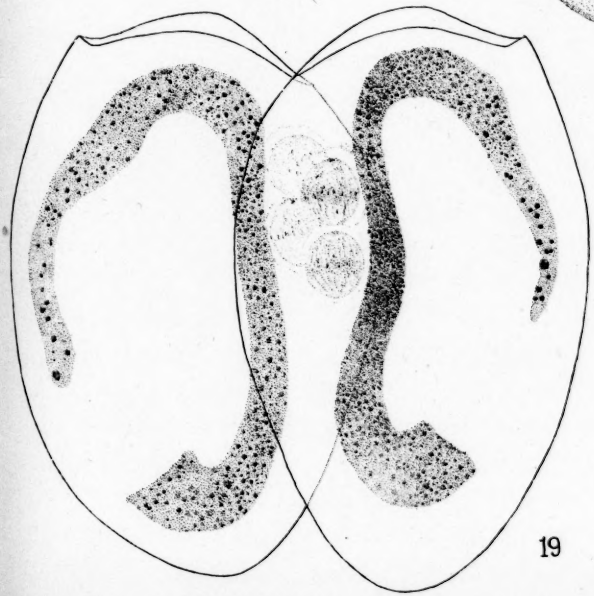

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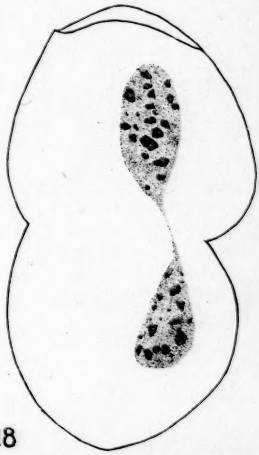

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results. If evaporation is much too rapid, the animals become motionless but are not fixed to the slide, as mentioned previously; and encystment does not follow. Fresh culture medium added at an early stage causes some animals to resume movement, others to disintegrate. If evaporation is less rapid, degrees of abnormal cysts are formed. The series of morphological changes previous to formation of a cyst wall appear to be reduced to one, and that a sudden, contraction. These cysts are smaller than normal, and they vary in shape from irregular and knotlike to a nearly normal form. In each case when cysts of these types were set aside after an addition of fresh culture medium, excystment occurred in a few of them. The resulting animals were abnormal in shape. Although most of these died a short time following excystment, one or two animals in each case became entirely normal in appearance after 3 or 4 days.

CYTOLOGICAL DETAILS

A study of more than three hundred permanent mounts of cysts in various stages substantiates the observations already mentioned, that at some stage there is no evidence of external organelles. The rôle of the nuclei could not be observed in the living condition due to the thickness of the cyst. In the earliest stage (Plate III, Fig. 14) of encystment, stained specimens showed both a micronucleus and a macronucleus, the appearance of which is similar to those found in free-swimming organisms not undergoing division. In the cytoplasm membranelles can be distinguished as a striated region, and also cirri can be traced to some extent. At another and later stage, nuclei show a similar structure as stated above, but the region of the membranelles is now seen as a field of homogeneous material bordered on the periphery by an abrupt beginning of coarsely granular cytoplasm.

Cysts approximately 4 hours old (Plate III, Figs. 16, 17) show a decided change in the condition of the nuclear portion. A micronucleus can be found in the majority of cases, although occasionally stained but lightly (Plate III, Fig. 17). The macronucleus consists of darkly staining bodies within a substance less marked in staining properties. Strengthening the supposition that this condition of the macronucleus indicates a beginning reorganization of the nuclear apparatus are the facts that macronuclei with essentially the same

structure have been found in stained preparations of free-living animals undergoing division (Plate III, Fig. 18) and at a stage in conjugation (Plate III, Fig. 19). At another and later stage, the macronucleus is in process of fragmentation.

An interesting condition is found in the macronucleus during excystment (Plate III, Fig. 20). The chromatin is coarsely granular except at either end, where spherical masses of chromatin are dispersed through a less stainable medium. The whole is inclosed within a fairly distinct nuclear membrane.

DISCUSSION

From the evidence already given, there can be no doubt that a reorganization process takes place during encystment in *E. taylori*, but the exact procedure and time at which the changes may occur has as yet not been definitely established. The method whereby both encystment and excystment may be induced makes possible a more detailed study, which is now in progress.

Regarding the external organelles, their disappearance and reappearance at some stage during encystment, the following statements may be made. Two cysts which had reached the stage when a cyst wall was well defined, but in which membranelles had not yet disappeared, were caused to excyst by the addition of slightly more than the usual amount of tap water for this process. In each of these excysted forms a complete (apparently the old) set of membranelles and cirri were present, and *these were accompanied by outgrowing buds*.

Reorganization includes the nuclear apparatus. Early cysts without exception contain a macronucleus, the appearance of which is the same as that found in free-swimming organisms not undergoing division. Cysts 4 hours old or older show a variety of conditions of the nuclear portion directly comparable to stages found in binary fission and conjugation. A macronucleus such as is found in the first stage may be present in some of these, which may mean that a dedifferentiation and redifferentiation process had already taken place. It is conceivable that the rate at which reorganization takes place may vary with the individual organisms and may depend upon the conditions present in that organism prior to encystment. The continuance of encystment in spite of conditions favorable to excystment, as previously described, is also significant.

SUMMARY

This study of *Euplotes taylori*, sp. nov., has brought out the following facts:

1. Encystment may be induced at will by a gradual evaporation of the culture medium, probably within certain limits of temperature range.

2. Cysts are ellipsoidal in shape—40–55 microns in diameter and approximately 20–28 microns in depth.

3. Abnormal cysts or death may result when the rate of evaporation is greater than that ordinarily produced at 22° C.

4. Excystment may be brought about by addition of fresh culture medium or by a smaller amount of distilled or tap water.

5. During encystment *E. taylori* undergoes a series of morphological changes which include dedifferentiation of external organelles.

6. With excystment a redifferentiation process takes place.

7. There is evidence of nuclear reorganization during encystment. This reorganization is similar to that taking place during binary fission and conjugation in this species.

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THE PHYSIOLOGY OF THE CRUSTACEAN HEART. I. THE EFFECT OF VARIOUS IONS UPON THE HEART RHYTHM OF THE CRAYFISH, CAMBARUS CLARKII

(Nine figures)

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INTRODUCTION

PROBLEMS concerning the relationship of various ions of the blood to the normal heart rhythm of crustaceans have received relatively slight consideration in comparison to the abundance of data available for certain other groups of cold-blooded animals. Moreover, the bulk of investigation upon the crustacean, with respect to the effect of ions, has dealt chiefly with the larger salt-water forms, notably the crabs and lobsters. In this connection attention is called to the following investigations: Rogers (1905), working upon the crab (*Brachynotus nudus*), found that Na, K, and Ca each have an important effect upon heart contraction. Hogben (1925), comparing the effect of ions upon the hearts of *Maia* and *Homarus*, found that there was a variation in the ionic requirement of the two species in that the concentration of K present in the perfusing solution determined whether or not the lack of Ca would produce systolic or diastolic arrest. He found, however, that an excess of K produced systolic arrest of the heart in both species. Hogben furthermore found that the hearts of both of these crustaceans were relatively insensitive to changes in H-ion concentration within the range of pH 5.5–7.2.

Comparatively few similar observations have been made on the fresh-water crayfish heart, although it provides favorable material for such an investigation. Hoffmann (1912), in the course of his investigation on action currents in the crayfish heart (*Astacus*), noted that an excess of either K or Ca produced little effect upon the normal heart rhythm. His chief concern in these ions lay only in finding a suitable perfusing solution for maintaining the normal heart pulsations.

An analysis of crayfish blood (*Astacus*) by Fredericq (1885), indicates a surprisingly low percentage of soluble salt content compared to the concentration determined for many marine crustaceans. In this connection it was found that the soluble salt content of *Astacus* was less than 1 per cent, while that of Palinurus and Homarus amounted to approximately 3 per cent. This suggests the probability that the ionic requirements for the maintenance of normal heart rhythm in the fresh-water crayfish might be somewhat different from that found true for salt-water species. It was with this idea in mind that experiments were planned to determine the effect of various ions, particularly Na, K, Ca, and Mg, upon the heart rhythm of the crayfish; and secondarily, to record any peculiarities or similarities due to ionic action as have been observed for other crustaceans.

It is with pleasure that I acknowledge my indebtedness to Dr. O. M. Helff for suggesting the problem and for his kindly interest and criticism throughout the course of the investigation.

MATERIALS AND METHODS

The crayfish employed for all experiments was *Cambarus clarkii*, a shallow-water species native to the swamps and fresh-water marshes of Louisiana. They are a very strong and active variety, ranging from 3 to 5 inches in length. The animals were shipped from Louisiana and upon arrival were placed in a large tank of running water for several days before being used for experimental purposes. The purpose of this precaution was to assure the animals' complete recovery from possible disturbances due to shipping, and also to acclimate them to any change in the environment.

The technique employed in obtaining graphic records of the effect of various ions on heart rhythm was briefly as follows: A piece of the carapace about 1 cm. square was removed from an area directly over the heart. This exposed the gills and the heavy body wall separating the carapace from the pericardium. A portion of the body wall at this point was then dissected away very carefully and the pericardium exposed. The pericardium was then partially removed, thus exposing the heart. The animal was now placed in a clamp which held it securely in a horizontal position and prevented

the movements of the appendages from interfering with the accuracy of the recording apparatus.

The recording apparatus consisted of the usual set-up, including a kymograph, heart lever, and signal magnet, the latter operated by means of an electrical clock timing apparatus. The comparative weakness of the heart pulsations of the crayfish necessitated the use of a heart lever which would reduce friction to a minimum. For this purpose a broom straw, balanced upon a very fine pivot, was employed. The heart was then attached to the lever by means of a very fine wire and steel hook, the latter being carefully inserted through the dorsoposterior region of the heart in order that the normal functioning of the ostia might not be impaired. The pulsations were then recorded upon a slowly moving drum.

It was found desirable to keep the heart perfused at a constant rate for all solutions employed. For this purpose a series of delivery siphons was arranged, one for each solution, whose rate of flow could be accurately regulated by means of screw clamps. The perfusing solutions were allowed to run into the pericardial sinus, from which point they were quickly perfused through the heart by way of the ostia. With this system of siphons it was possible to change the perfusing solutions very rapidly, while the temperature of the solutions could be checked continuously. The temperature of the perfusing solutions was maintained at $25 \pm 0.1^\circ$ C., which was generally the room temperature and also that of the aquarium water in which the stock animals were kept.

A series of perfusing solutions containing varying concentrations of NaCl, KCl, $CaCl_2$, and $MgCl_2$ were tested in an attempt to secure a perfusing bath capable of maintaining normal heart rhythm for a considerable length of time. In this connection Hoffmann's (1912) double-Ringers solution was tried, but it was found inadequate in maintaining normal heart rhythm for the species in question. It was finally found that a solution containing 1 per cent NaCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$ produced no noticeable effects on the normal rate, amplitude, or tone of the heart pulsations, even when applied over a period of several hours. This solution was employed as a control bath and as a basis for observing the effect of solutions containing different ionic concentrations. For con-

venience, the foregoing perfusing solution will be spoken of as the "normal perfusing solution" throughout the present paper and will be designated by *N* on all graphic records. The normal perfusing solution and all other ionic solutions were made with double-distilled water.

The experiments consisted in first perfusing the intact heart for several minutes with the normal perfusing solution. This removed most of the blood, not only from within the heart, but also from the surrounding pericardial sinus. After the rhythm of the heart had become normal, the normal perfusing solution was then discontinued, the ionic solution to be tested immediately applied at the same rate, and changes in heart rhythm recorded graphically.

In order to determine whether the various solutions employed actually entered and perfused the heart through the ostia, the following simple test was applied: A small quantity of powdered carmine was added to the perfusing solution being applied to the heart. By means of a hand lens, it was quite evident that the carmine granules were being sucked into the heart through the ostia, thus proving that the solutions applied to the surface of the heart are quickly perfused through that structure.

RESULTS

The effect of the Na ion.—The heart of the crayfish was found to beat normally for several hours when perfused with a solution containing 1 per cent NaCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$, but upon removal of the KCl and $CaCl_2$, a rapid decrease occurs in the diastolic tone, with an increased amplitude of the beats (Fig. 1). There is always a slight acceleration in the rate at first, but after 10 or 15 minutes the beats become less regular and the heart shows signs of typical sodium fatigue, eventually stopping in diastolic arrest. The arrest is generally brought about in the intact heart in less than 30 minutes. Upon doubling the NaCl in the normal perfusing solution, a slight decrease in diastolic tone follows, and also the characteristic increase in amplitude (Fig. 2). The rate is somewhat greater as compared with the results obtained when perfusing with a pure NaCl solution. Upon application of the normal perfusing solution, the normal rate and amplitude are first restored (Fig. 2); and later on, the normal tone. Upon the application of a

solution containing 0.028 per cent KCl plus 0.024 per cent plus $CaCl_2$ an immediate decrease in tone occurs, followed shortly by a sharp and continued increase in tone (Fig. 3). With continued perfusing, the rhythm is greatly depressed, the amplitude falling markedly until the heart goes into diastolic arrest. Subsequent application of a

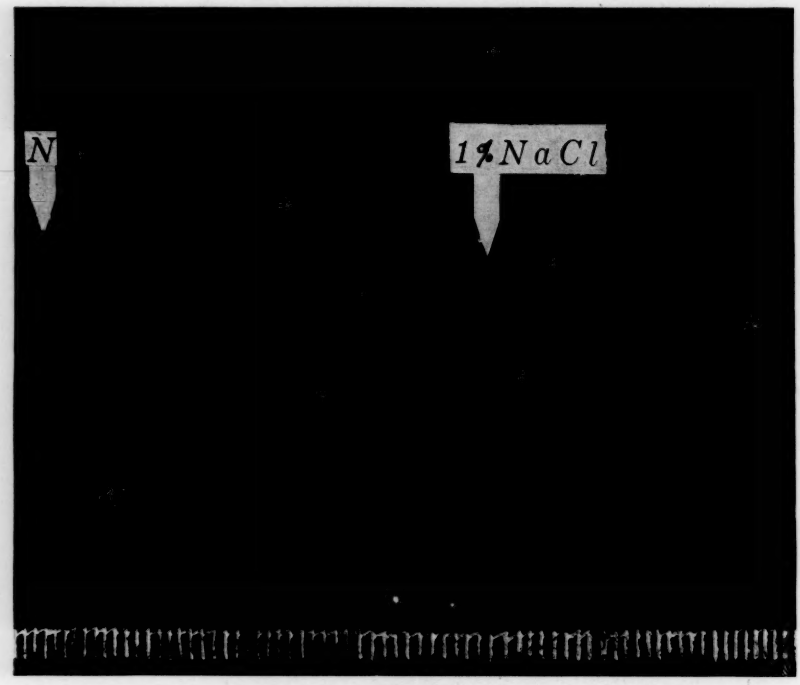

FIG. 1

normal perfusing solution results, in time, in complete recovery of the normal rhythm.

The effect of K and Ca ions.—Upon applying a 0.024 per cent $CaCl_2$ solution to the heart, a sharp increase in tone immediately follows, coupled with an irregularity in the rhythm (Fig. 4). The beats are not all of the same amplitude, and there is a decrease in the rate. If the $CaCl_2$ is increased in the normal perfusing solution, the

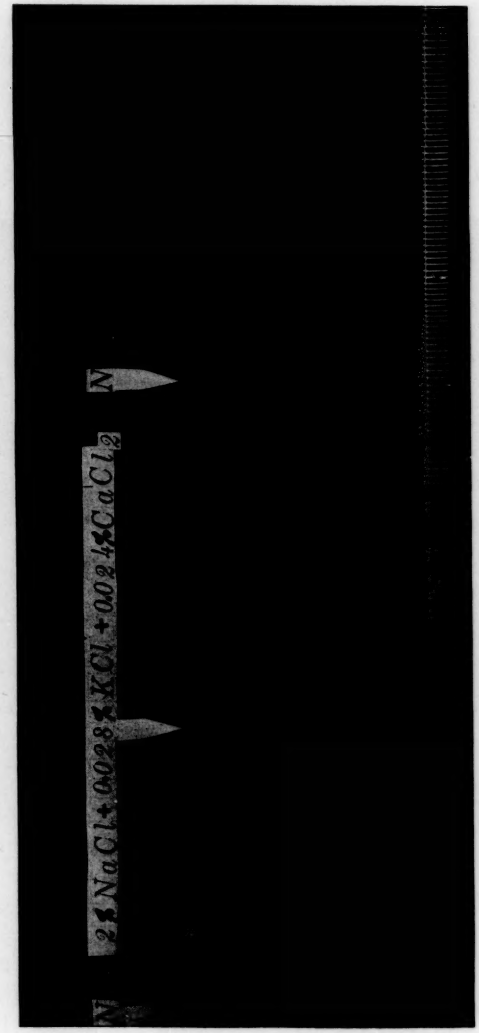

FIG. 2

rate is greatly decreased; but only slight changes in the tone and amplitude of the beats can be noted at first. Figure 5, for example, represents the depressor action on a heart perfused with a solution containing 1 per cent NaCl plus 0.028 per cent KCl plus 0.1 per cent $CaCl_2$, and in which the $CaCl_2$ concentration is approximately four times greater than in the normal perfusing solution. If the heart is perfused with a solution lacking in Ca, it will beat normally for some

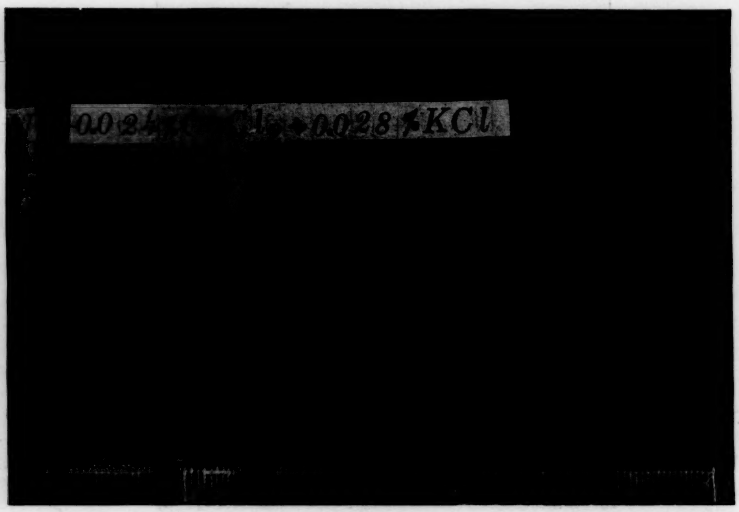

FIG. 3

time. Some hearts will continue beating for as long as $2\frac{1}{2}$ hours when perfused with a solution containing 1 per cent NaCl plus 0.028 per cent KCl, with but very slight variation in the normal rhythm. At the end of this time, short periods of systolic arrest occur, the heart finally stopping in systole. Subsequent perfusing with a normal perfusing solution for 10 minutes invariably failed to initiate pulsations.

The effect in absence of KCl is somewhat different. The use of a solution from which KCl is absent, results in complete stoppage of the heart in less than $\frac{1}{2}$ hour. Figure 6A is a record of a heart perfused with a normal perfusing solution, while Figure 6B represents the type of pulsations obtained at the close of a 25-minute applica-

tion of a solution containing 1 per cent NaCl plus 0.024 per cent $CaCl_2$. The heart, it will be noted, gradually increases in tone; while the relaxation period of each beat is greatly prolonged, resulting finally in systolic arrest. Upon the subsequent application of the normal perfusing solution, the normal rhythm is gradually restored.

A pure 0.028 per cent solution of KCl was found to have a depressor action on both the amplitude and rate, although the tone

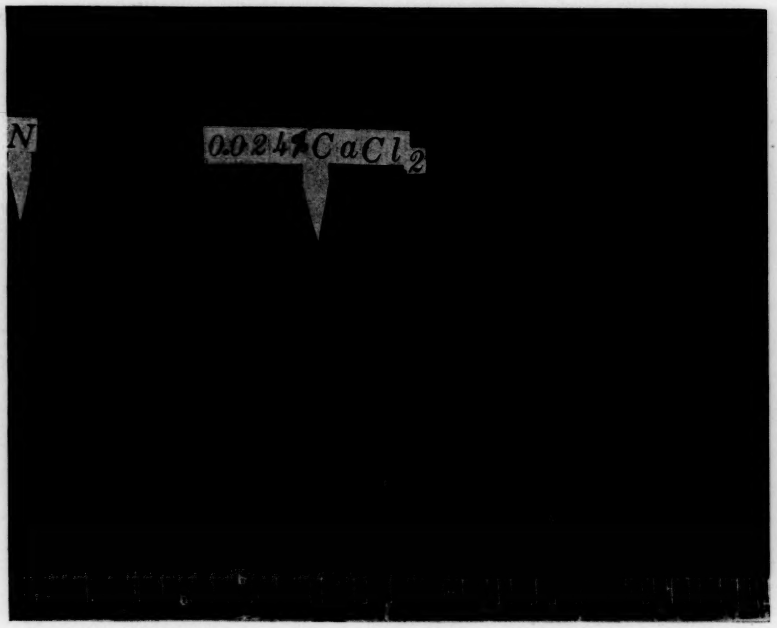

FIG. 4

appeared to be slightly increased. Complete relaxation of the individual beats is slightly prolonged with continued perfusion, resulting finally in systolic arrest. If the concentration of KCl is increased slightly in the normal perfusing solution, no noticeable change in rhythm can be detected. If, however, it is increased to 0.1 per cent, there is a characteristic decrease in the rate and amplitude of the beats coupled with a slight increase in tone. In addition, abnormal

rhythm, composed of several small beats superimposed upon each large beat, is obtained (Fig. 7).

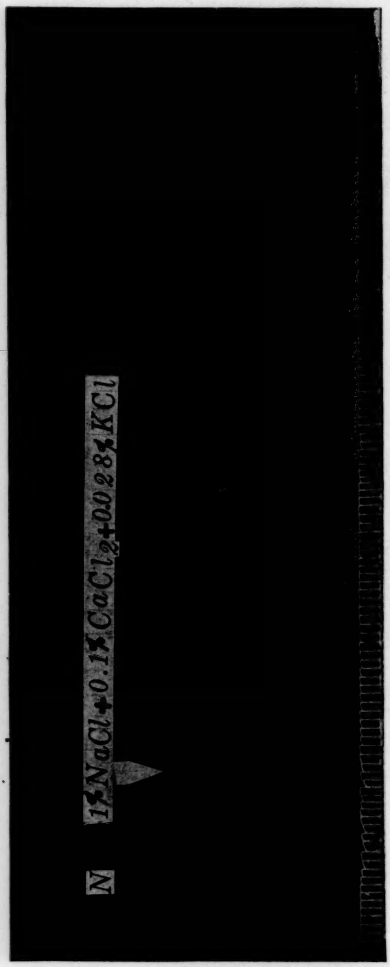

FIG. 5

The effect of the Mg ion.—The heart of the crayfish beats normally in the absence of $MgCl_2$, while a slight amount in the normal perfusing solution produces no noticeable effects. A normal perfusing solution for example, to which has been added 0.02 per cent $MgCl_2$, produces no specific effect on the normal heart rhythm; and the heart will continue beating, on the average, for some length of time. If the $MgCl_2$ is increased, however, to 0.1 per cent, the rate is considerably decreased, although the tone and amplitude are but slightly changed.

The effect of the hydrogen ion.—The normal perfusing solution and the ionic solutions as used in determining the effect of various ions on heart rhythm in the present work had a pH of $7.0 \pm .01$. It seemed desirable to determine whether or not slight changes in the pH of the solutions employed may have had an effect upon the heart rhythm. To determine this, normal perfusing solutions were em-

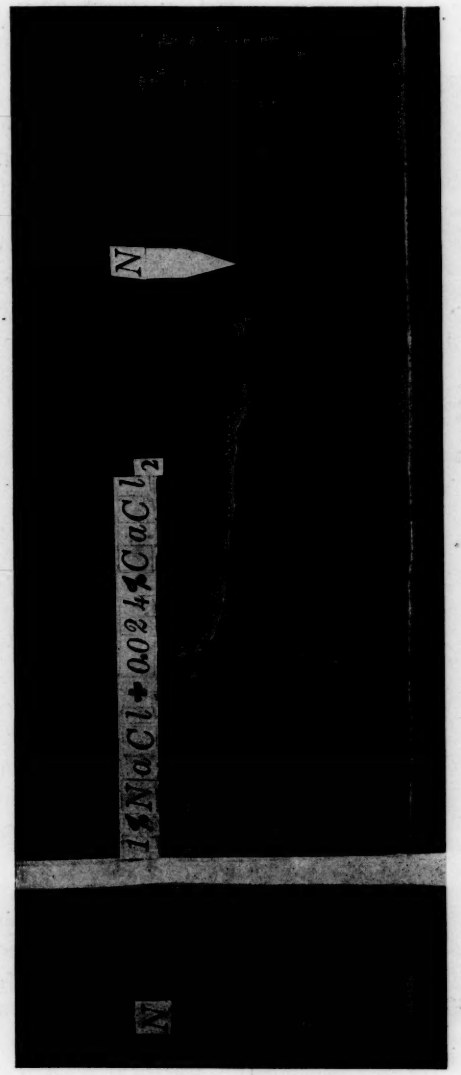

FIG. 6B

FIG. 6A

ployed whose pH had been lowered by means of CO_2 and HCl. It was found that the pH of the normal perfusion solution could be dropped suddenly from 7.0 to 5.5 without any appreciable change in the rhythm; but if dropped below 5.5, an increase in tone resulted (Fig. 8). It was also found that if the pH was dropped to 5.0, or any point below, the heart was immediately thrown into systolic arrest (Fig. 9). An interesting phenomenon was also noted when the pH of the perfusing solution was gradually lowered. The heart rhythm under these conditions remained normal when perfused down as low as pH 4.0, but was finally and gradually thrown into peculiar tone waves similar to those noted in a dying heart. It would appear, therefore, that the crayfish heart is able to adapt itself to relatively high H-ion concentrations, providing the pH is lowered gradually.

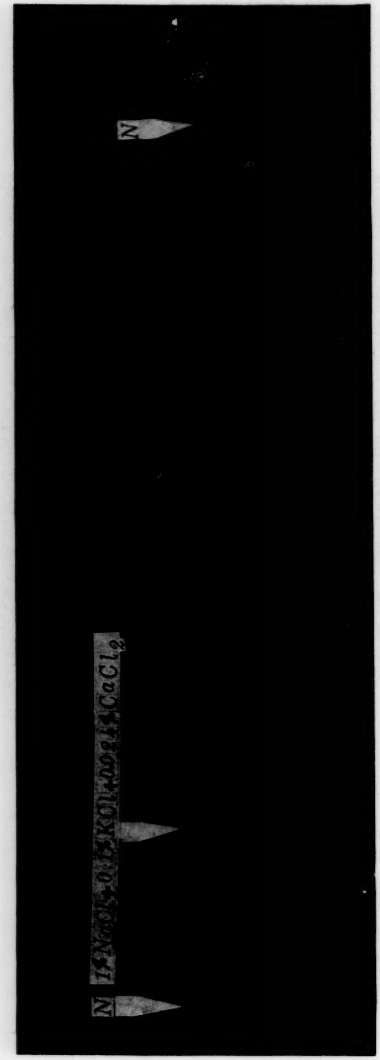

FIG. 7

By means of NaOH, the pH of the perfusing solution could be increased without changing the Na content greatly. It was found that such solutions having a pH up to at least 9.0 had little or no effect upon the normal heart rhythm.

DISCUSSION

A question might well be raised regarding the ionic concentrations of the perfusing solutions employed, as compared with the various known ionic concentrations of the crayfish blood. An analysis by Dohrn (1866) gives the following approximate percentage composition of *Astacus* blood: NaCl, 0.81 per cent; KCl, 0.28 per cent: $CaCl_2$, 0.47 per cent; and $MgCl_2$, 0.09 per cent. Griffith (1892), work-

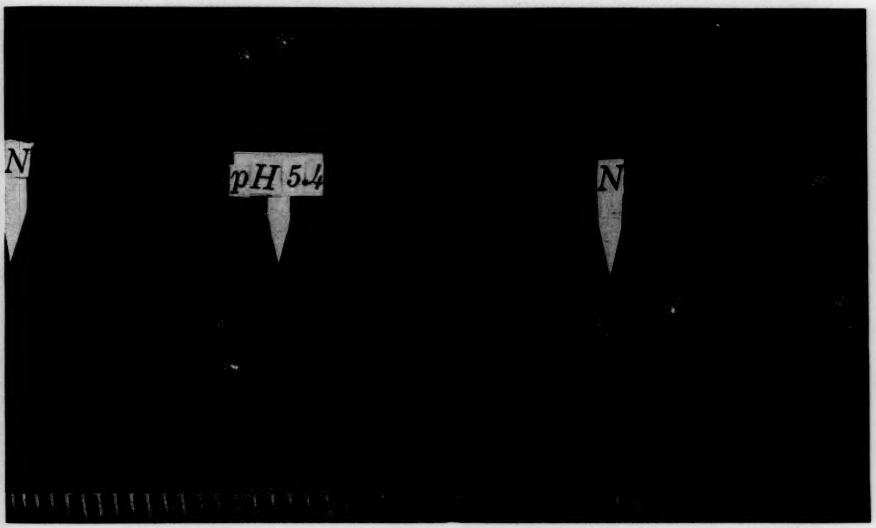

FIG. 8

ing on the same species, quotes somewhat lower concentrations of K and Ca, although the Na content was found to be approximately the same. The ionic concentrations of the perfusing solutions employed in the present work (with the exception of the Na) were much lower than those determined for *Astacus* blood. It was found that a perfusing solution, identical in ionic concentrations with those of *Astacus* blood, would not maintain the normal heart rhythm of *Cambarus clarkii*. However, if the concentration of NaCl was increased to 1 per cent and the KCl and $CaCl_2$ decreased to 0.028 per cent and 0.024 per cent, respectively, a solution was obtained which would maintain normal heart rhythm for a period of several hours.

A normal perfusing solution containing less than 1 per cent NaCl, operated to decrease the normal rate of the beats. The foregoing results, therefore, tend to indicate that the ionic content of the blood of *Cambarus clarkii* is somewhat different from that as determined by Dohrn for *Astacus*. Furthermore, the strengths of the ionic con-

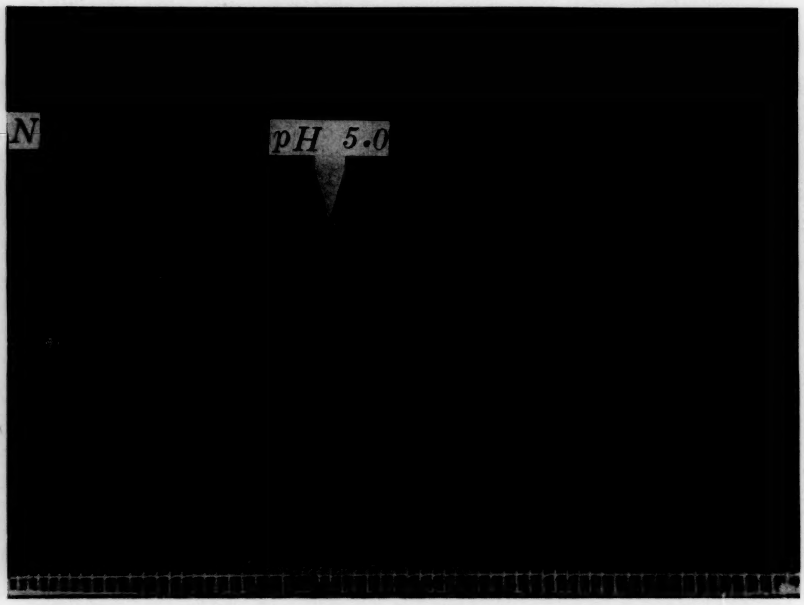

FIG. 9

centrations employed were such that the results obtained upon perfusion could not be attributed to toxic effects.

There has been considerable difference of opinion as to the rôle performed by Na, K, and Ca ions in the maintenance of heart tonicity in various animals. Ringer (1887), for example, working with the frog, noted that K and Ca ions displayed antagonistic properties in this regard. He found that an excess of the former ion decreased the tone, while the latter tended to increase it. Howell (1901), working with the terrapin, states that the antagonism is principally between the Na and Ca and that K exercises but a slight

influence. The results upon the intact heart of the crayfish show quite clearly that the Na and Ca ions are undoubtedly concerned in maintaining the tone. Furthermore, there are indications that K also has an important influence upon the tone. It has been shown that a heart perfused with 1 per cent NaCl invariably loses tone; but if perfused with one containing 1 per cent NaCl plus 0.028 per cent KCl, this pronounced loss in tone does not occur. It appears, therefore, that K, likewise, may have an antagonistic effect toward the Na ion.

It is also a question as to which of the three ions is most influential in the maintenance of the normal tone. Data have been presented which indicate that all exert some influence. The Ca and Na ions undoubtedly exhibit marked antagonistic effects, the former increasing and the latter decreasing the tone. The influence of the K ion upon the tone is somewhat problematic. It is undoubtedly not antagonistic to Ca in that a perfusing solution containing approximately equal ionic concentrations of the two causes a sharp increase in heart tone. It appears likely, therefore, that K and Ca exhibit similar properties in this respect and are each capable, to a greater or less degree, of antagonizing the effect of the Na ion. The difference in ionic concentration of the $CaCl_2$ and KCl necessary to completely antagonize the effect of a 1 per cent NaCl solution on the tone would indicate that the Ca ion exerts a stronger antagonistic influence than does the K ion.

That the Na ion exerts a distinctly opposite effect upon the rate and amplitude of the crayfish heart beat to that of the K or Ca ion appears quite evident. In every case (whether in a pure solution or in an excess in the normal perfusing solution) the Na was found to increase the rate and amplitude of the beats. This increase in rate and amplitude was probably due to the well-known irritating effect of the Na ion. Carlson (1909) noted that NaCl caused excitation of *Limulus* heart muscle in that the amplitude was increased. Hogben (1925), working upon the heart of *Maia*, however, observed that a pure solution of NaCl produced an almost immediate increase in tone, resulting shortly in systolic arrest. The results obtained by the latter worker, it will be noted, are not typical of the usual NaCl effect upon heart muscle. The results obtained for K and

Ca ions upon the crayfish heart, as regards rate and amplitude, are characteristic of the effects of these ions on heart muscle in general, in that the rate and amplitude are depressed.

The possible influence of the Mg ion on normal heart rhythm is interesting to consider at this time. Fredericq (1913), for example, noted that Mg was not essential in maintaining the heart rhythm of the octopus. Mines (1911), on the other hand, found Mg was very essential for the normal heart rhythm in *Pecton*. Clark (1927) states that, in general, the presence of Mg is not essential for the normal heart action of non-marine forms. There appears to be, therefore, considerable variation in the response of different invertebrate hearts to the Mg ion. In this respect, the crayfish heart was found to beat normally in the total absence of Mg. Moreover, slight traces in the normal perfusing solution had no marked effect. The results obtained for the crayfish, therefore, indicate that the Mg ion is not essential for the maintenance of normal heart rhythm.

An interesting condition exists in the crayfish heart in that it is quite insensitive, within certain limits, to changes in H-ion concentration. Normal heart rhythm, it will be recalled, was obtained when perfusing with solutions having a H-ion concentration as low as pH 5.5. A comparatively high pH (up to 9.0) likewise failed to evoke any irregularities in the normal rhythm. It will also be recalled in this connection that when the pH was lowered gradually (0.5 of a pH at a time), the heart could be made to respond normally under a much lower pH than if the drop in the pH had been made rapidly. In fact, normal rhythm was obtained by this method when the pH of the perfusing solution had been reduced as low as 4.0. Hogben (1925), in the course of his investigations upon the hearts of *Homarus* and *Maia*, likewise found that the hearts of these species responded normally down to a pH of 5.6. It would appear, therefore, that the crustacean heart may be relatively unaffected during rather extreme changes in H-ion concentrations. Whether this is an adaptation to the normal fluctuating H-ion concentration of crustacean blood or whether it only indicates an independence of normal heart rhythm so a relatively constant H-ion concentration of the blood, is an interesting question. It would seem profitable, in this connection, that a careful study be made of the H-ion characteristics of crustacean blood during consecutive periods of time.

SUMMARY AND CONCLUSIONS

1. A method was devised whereby the pulsations of the intact crayfish heart could be recorded and studied, graphically, while being perfused with various ionic solutions.

2. Various concentrations of NaCl, KCl, and $CaCl_2$ were tested in the perfusing bath in an attempt to find a solution which would maintain the normal heart rhythm. It was finally found that a solution containing 1 per cent NaCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$ fulfilled this purpose, the heart beating normally for several hours when exposed and perfused at a temperature of $25 \pm 0.1°$ C.

3. In an attempt to determine the effect of the Na ion upon the heart rhythm, three perfusing solutions were employed: One consisting of 1 per cent NaCl; another of 2 per cent NaCl plus 0.028 per cent KCl plus 0.024 per cent $CaCl_2$; and the third of 0.028 per cent KCl plus 0.024 per cent $CaCl_2$. Both solutions containing Na increased the normal rate and amplitude although decreasing the tone. The third, in which Na was absent, decreased the normal rate and amplitude but increased the tone markedly. It is concluded that the Na ion is undoubtedly concerned in regulating the tone of the crayfish heart.

4. Similar experiments were performed with solutions of KCl and $CaCl_2$. A pure solution of $CaCl_2$ produces a sharp increase in the tone and a slight decrease in the rate. Increasing the $CaCl_2$ in the normal perfusing solution affected the rate only, the latter being decreased slightly. A pure solution of 0.028 per cent KCl invariably depressed the rate and amplitude but had slight, if any effect, upon the tone. The removal of K from the control perfusing solution resulted in systolic arrest of the heart in less than 30 minutes. Normal rhythm could subsequently be restored upon the replacement of KCl in the perfusing solution. In general, therefore, it may be said that K and Ca ions depress the rate and increase the tone of the crayfish heart, the Ca ion having an especially pronounced affect upon the tone.

5. The addition of small amounts of $MgCl_2$ to the normal perfusing solution produced no change in normal heart rhythm. When a large excess was added, however, a marked decrease in the rate was recorded. The presence of Mg in the perfusing solution does not

appear, therefore, to be essential for the maintenance of normal heart rhythm. Furthermore, it does not seem probable from the results obtained, that concentrations of $MgCl_2$, as determined by analysis of crayfish blood, could have any marked effect on the maintenance of normal heart rhythm.

6. Normal perfusing solutions in which the H-ion concentrations had been altered by means of HCl, CO_2, and NaOH showed no marked effect upon normal heart rhythm within a range of pH 5.5–9.0. Decreasing the pH below 5.5 results in a marked increase in tone. It is concluded that the crayfish heart is capable of maintaining its normal rhythm under a considerable range of H-ion concentration and is not, therefore, peculiarly adjusted to function normally only when under the influence of a constant blood pH.

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THE REGULARITY OF RESPIRATORY MOVEMENTS OF LOCUSTIDAE

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IN RECENT years experiments have been recorded in which the rate of respiratory movements of insects has been used as a basis for the calculation of data concerning certain metabolic activities (Crozier and Stier, 1925). In order to test the validity of such a procedure, at the suggestion of Dr. Bodine of the Department of Zoölogy, University of Pennsylvania, a series of experiments designed to ascertain definitely the regularity of respiratory movements in grasshoppers was performed.

The insects were reared in cages in the laboratory and fed lettuce. *Melanoplus differentialis* was the species most extensively used; but a few *Melanoplus femur-rubrum*, *Arphia xanthoptera*, and *Chortophaga viridifasciata* were included.

In carrying out an experiment the grasshopper was placed in a small paper cylinder in which a hole had been cut so that the rise and fall of the abdominal segments might be noted. Practically no light reached the insect's eyes, and random bodily movements due to this type of stimulation were considerably reduced both in frequency and violence. In several experiments, with the hope of eliminating body movements entirely, the animal was placed ventral side down on a mirror and the abdominal movements reflected into the microscope by means of a second mirror. But the results were no more satisfactory than in those experiments in which the grasshopper rested dorsal surface down.

In a number of cases the grasshopper was decapitated and the rate then secured compared with the rate recorded for the same individual before decapitation. The variability of the rate after decapitation was found to be very similar to that before decapitation, and attempts to secure a constant rate by this method were soon abandoned. The respiratory rates of a grasshopper before and after decapitation are shown in Figure 1.

Observations were made with a binocular microscope, and the time required for ten respiratory movements taken with a stop watch. When the animal moved, readings were discontinued until all struggling had ceased and the breathing had once more become regular. The temperature of the room in which the experiments

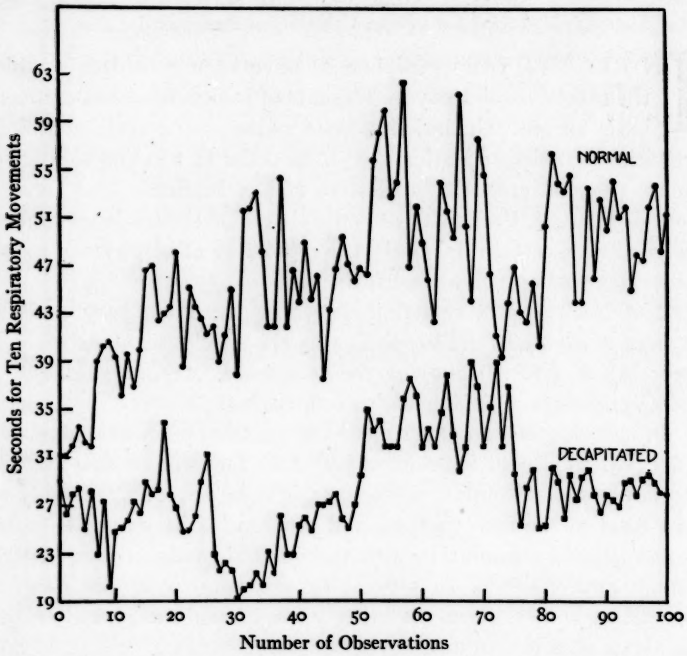

FIG. 1.—Comparison of rate of respiratory movement of a *Melanoplus differentialis* before and after decapitation. Experiments begun 15 minutes and 1 hour, respectively, after preparation of animal for examination.

were carried out varied $\pm 3°$ from an average of 21°, being practically constant on any one day. Records at short intervals of the temperature variations were kept in a number of the experiments, but the results obtained were such that this procedure was accounted unnecessary.

Series of a hundred or more readings were secured in nearly every case for each insect studied, and in all twenty-four grasshoppers were used, giving approximately three thousand observations.

The results of a typical experiment are shown in Figure 2. Readings were begun immediately after placing the grasshopper in the paper, and continued over 4½ hours. As usual only those readings were recorded which were taken during periods when the animal remained quiet. The breathing at first was rapid. This is as would be expected considering the unavoidable excitation of the insect attending such handling as was necessary in preparing it for examination. But the slowness with which the effects of this stimula-

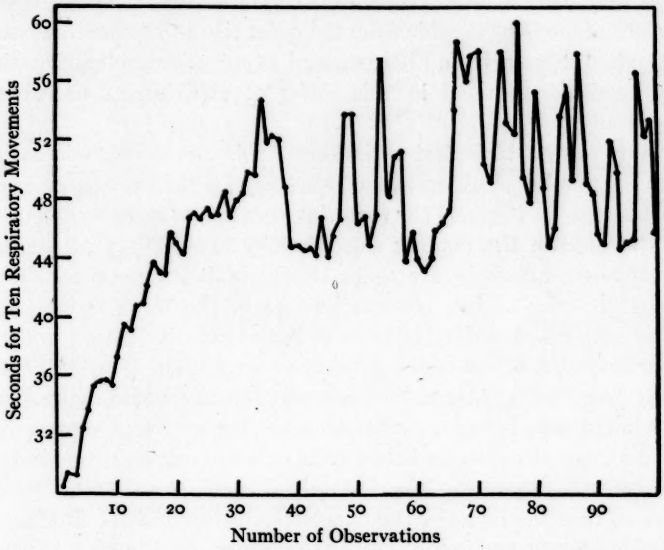

FIG. 2.—Respiratory movements of a female *Melanoplus differentialis*, showing stimulation of rate due to handling and consequent slowing of rate as effect gradually wears off. Experiment begun 15 minutes after preparation of animal for examination.

tion wore off is remarkable, for almost an hour was required before the respiratory rate reached what might be called a level. The duration of this slowing-down process varied with the individual studied but was usually between a half-hour and an hour. Experiments, therefore, in which the respiratory movements of grasshoppers are used as an index of physiological changes would be of little value unless considerable time was allowed to elapse between the handling of the animal and the taking of the first observation.

In an experiment with a female *Melanoplus differentialis* in which 220 observations were secured the difference between the highest and the lowest readings was 9.2 seconds. In one other case, shown in Figure 3, a *Melanoplus femur-rubrum* gave 100 readings between 21.8 and 31.6 seconds. With every one of the other animals used, however, the variations were much greater than this. A male *Melanoplus femur-rubrum*, for instance, gave readings ranging from 21.5 to 67.5 seconds during the period of an hour in which the temperature remained constant to $\pm 0.5°$, and the animal showed no signs of movement aside from the quiet rise and fall of its ventral abdominal segments. In Figures 4 and 5 typical examples of respiratory rates as obtained in this series of experiments are shown graphically.

With normal biological variations of this sort the question arises as to the number of observations necessary for the drawing of sound conclusions. In Figure 6 the material from an entire experiment has been treated in the manner suggested by Scott (1927). The individual observations in the order in which they were secured, the means of series of five observations each, the mean deviation of the means of these series, the mean of the cumulative series, and the mean deviation of the cumulative series are shown. The final mean of the 100 readings is carried back through the entire figure. This experiment was begun 15 minutes after the grasshopper was prepared for examination, and the effects of handling on the respiratory rate are still apparent. The low initial readings affect the entire series so that the mean of the cumulative series and the final mean coincide only at the end of the 100 readings. In Figure 7 the first 25 readings, obtained before the so-called "level" was reached, are omitted; and the remainder treated as were the 100 in Figure 6. Here the mean and the mean deviation of the cumulative series become relatively constant after 40–50 observations have been taken. It is evident, then, that less than 40 determinations of this sort would be an insufficient number upon which to base conclusions.

The data from another experiment have been similarly treated in Figure 8, but series of 10 readings each are used instead of 5. The persistent effects of the excitement caused by handling, here too are clearly shown. In Figure 9 the first 20 figures have been omitted and the last 80 alone considered. After 40–50 readings have

been taken, the mean and the mean deviation of the cumulative series become fairly constant, again indicating that this number of

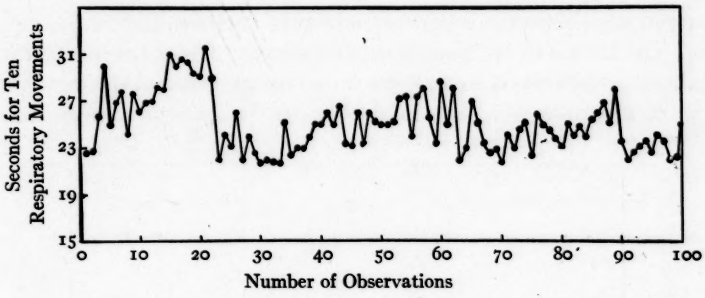

FIG. 3.—Rate of respiratory movement of a *Melanoplus femur-rubrum*, showing unusual regularity. Experiment begun 15 minutes after preparation of animal for examination.

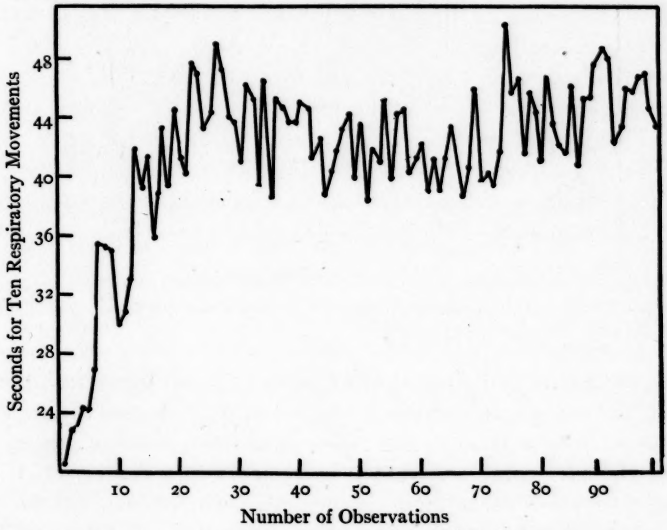

FIG. 4.—Respiratory movements of a female *Melanoplus differentialis*. Experiment begun immediately after preparation of animal for examination.

observations must be made before an average really representative of the series is obtained.

The range of variation in the experiments just analyzed is considerably less than that found with many of the grasshoppers studied, and with greater variation even more than 50 observations would be required in order to secure a stable mean.

The failure to obtain a more uniform rate is not at all surprising when the number of factors which may possibly affect the regularity of the breathing of insects is considered. Temperature undoubtedly

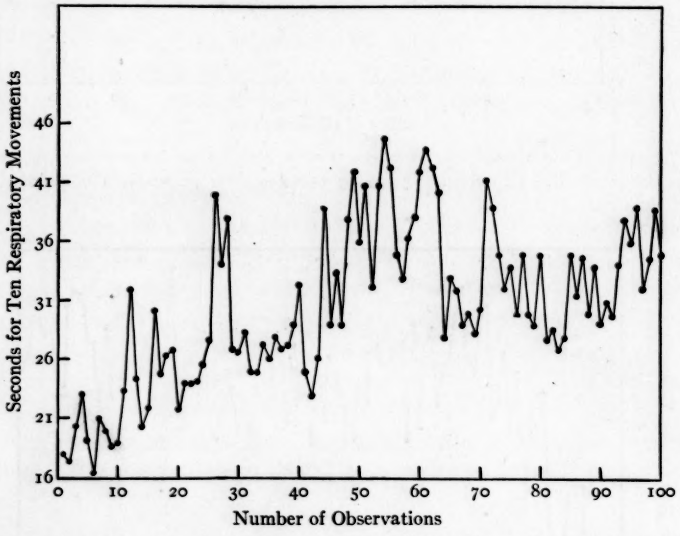

FIG. 5.—Rate of respiratory movement of a *Melanoplus femur-rubrum*. Experiment begun 15 minutes after preparation of animal.

does play a part, but since at any one temperature great variations occur, the other factors cannot be disregarded. Again, the movements made by a grasshopper—aside from those of breathing—will affect the succeeding respiratory movements to a degree which is roughly proportional to their violence. Thus the grasshopper which has struggled vigorously in attempting to escape while being placed in a paper cylinder will show the effects of this activity for perhaps an hour or longer. If, on the other hand, a grasshopper which has been resting quietly for a long time moves one of its legs, the

respiratory rate will rise sharply, but in a few moments will be back in the neighborhood of the rate immediately preceding the movement.

Another factor which must be considered is the depth of the respiratory movement. At times the rise and fall of the floor of the abdomen will be so slight as to be scarcely perceptible, and at others the movements—even though regular—will be so deep that they

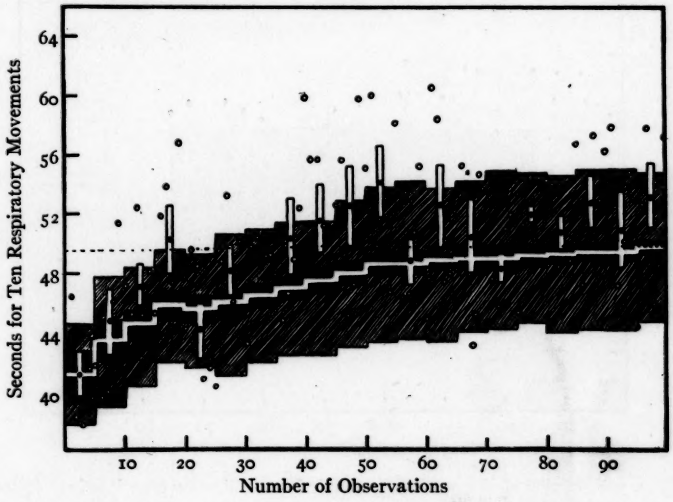

FIG. 6.—Rate of respiratory movement of a female *Melanoplus differentialis*, showing effect of excitement caused by handling on stabilization of mean and mean deviation of the mean of the cumulative series. The individual determinations (circles), the means of the successive series of fives (dots), the mean deviations of the means of these successive series (vertical bars), the mean of the cumulative series (horizontal white line), the mean deviation of the cumulative series (diagonally hatched field), and the mean deviation of the mean of the cumulative series (solid black field) are shown. The final mean is carried back through the figure as a horizontal dotted line.

can easily be recorded without the use of a microscope. Since the actual amount of air inspired and expired by the insect during any period is dependent upon both the depth and the rate of respiratory movement, neither alone can be taken safely as an index of respiratory activity.

The rate at which different grasshoppers breathe after comparative regularity has been established and at temperatures close to 21° varies with the individual animal. This fact has already been noted by Crozier and Stier (1925). The mean of 800 respiratory movements for one insect was 3.5 seconds; for another, 1.6; and for others such figures as 2.7, 3.8, and 2.5 were obtained. Possibly

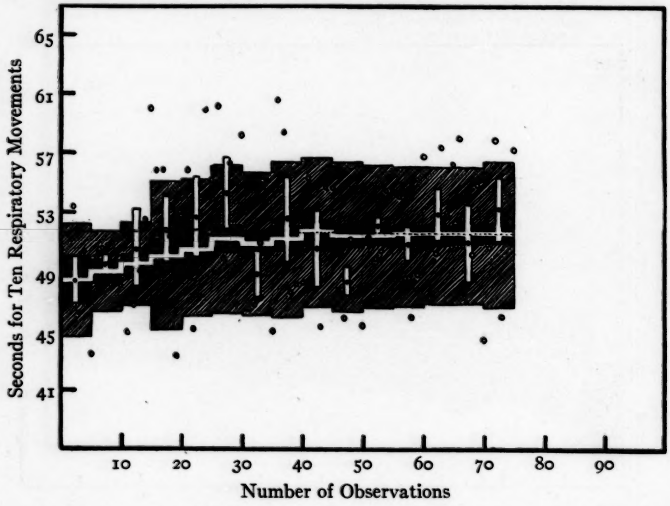

FIG. 7.—Number of observations necessary for stabilization of series of respiratory movements after "level" has been reached. Data from same experiment as Figure 6, but first 25 readings have been omitted. Symbols are same as those used in Figure 6. Here the mean and the mean deviation of the mean of the cumulative series becomes relatively stable after 40–50 observations have been taken.

such differences are dependent upon the health of the animal, the size, the time which has elapsed since the taking of food, etc.

CONCLUSION

The study of the influence of various factors upon the rate of respiratory movement in grasshoppers is not without its difficulties. The problem—at first sight a simple one—is in reality quite complex, and conclusions drawn from experiments in which the effects upon the respiratory rate of but one or a few of these factors are considered are of decidedly questionable value.

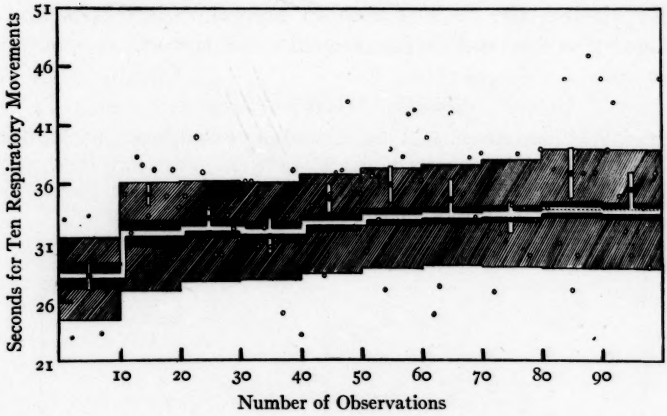

FIG. 8.—Rate of respiratory movement of a female *Melanoplus femur-rubrum*. Symbols and explanation the same as those used in Figure 6.

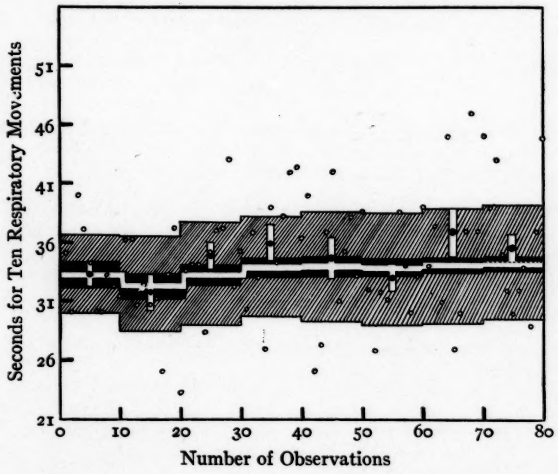

FIG. 9.—Rate of respiratory movement of a female *Melanoplus femur-rubrum*. Data from same experiment as Figure 8, but first 20 readings have been omitted. Symbols and explanation same as in Figure 7. Here the mean and the mean deviation of the cumulative series become relatively stable after about 40 observations have been taken.

In closing, the authors wish to express their thanks to Dr. Bodine for advice and encouragement given them in the course of their work.

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